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Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms

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ABSTRACT

Phosphodiester and phosphorothioate oligonucleotides in α and β configurations directed against the initiation codon region of the HIV-1 *rev* gene were evaluated for their ability to inhibit HIV-1 replication in acutely and chronically infected human CEM cells. Encapsulation in antibody-targeted liposomes (immunoliposomes) permitted intracellular delivery and distinction between oligonucleotide-mediated inhibition of viral entry and intracellular effects on viral RNA. Our results are consistent with four mechanisms of antiviral activity for these antisense oligonucleotides: (i) interference with virus-mediated cell fusion by free but not liposome-encapsulated phosphorothioate oligonucleotides of any sequence; (ii) interference with reverse transcription in a sequence non-specific manner by phosphorothioate oligonucleotides in α and β configurations; (iii) interference with viral reverse transcription in a sequence-specific and RNase-H-independent manner by α and β phosphodiester oligonucleotides; (iv) interference with viral mRNA in a sequence-specific and RNase-H-dependent manner by β-phosphorothioate oligonucleotides.

INTRODUCTION

Antisense oligonucleotides are of potential interest for clinical applications that exploit their antiproliferative and antiviral properties (reviewed in ref. 1). Native (phosphodiester) antisense oligonucleotides have generally been reported to have little (2) or no inhibitory effect against HIV-1 in culture (3–5) because they are rapidly degraded in culture medium (6–8). Two approaches exist to circumvent this degradation. One has been the development of various modified oligonucleotides with increased nuclease resistance (reviewed in ref. 9). These include phosphorothioate oligonucleotides and oligonucleotides with the sugar moiety oriented in the α configuration. Phosphorothioate oligonucleotides have been shown to inhibit the replication of HIV-1 *in vitro* by both sequence-non-specific (3,5,10,11) and specific processes (12–16), depending on the type of infection. In acutely infected cells, phosphorothioate oligonucleotides acted in a sequence-non-specific fashion probably by inhibition of viral binding and fusion (17,18) and/or viral reverse transcriptase (RT) (19,20). In chronically infected cells, however, only sequence-specific oligonucleotides are active, since after integration virus-cell binding and reverse transcription are no longer relevant (21).

The second approach used oligonucleotides associated with carrier systems. Oligonucleotides have been encapsulated in liposomes, including antibody-targeted liposomes (immunoliposomes), which protected them against nucleases and increased their delivery into cells (22,23). We and others have previously demonstrated the ability of liposome-encapsulated oligonucleotides to inhibit viral replication in cultured cells (22,24,25). The use of immunoliposomes permitted a direct comparison of the biological activity of phosphodiester and phosphorothioate anti-*rev* oligonucleotides delivered into acutely and chronically HIV-infected cells in a manner that eliminates the problem of nuclease degradation in the medium. The intracellular delivery of molecules encapsulated in liposomes of the same size and targeted to the same molecules should be identical. The antiviral activity obtained for both β-phosphodiester and phosphorothioate anti-*rev* oligonucleotides was very similar (25). This suggests that, in acutely infected cells, sequence-specific effects observed with phosphodiester oligonucleotides were quantitatively as important as non-specific effects seen for the phosphorothioates, such as inhibition of viral binding and inhibition of RT.

In chronically infected cells, in contrast to acute infection, antisense phosphorothioate but not phosphodiester oligonucleotides encapsulated in immunoliposomes inhibited HIV-1 replica-

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tion (25). The effect was sequence specific and dose dependent. The absence of activity of the phosphodiester oligonucleotides in this model probably reflects the greater amount of viral mRNA produced by the integrated virus than that present immediately after infection (26) and the sensitivity of phosphodiester oligonucleotides to intracellular nucleases.

The viral genomic RNA, RNA splice sites and viral messenger RNAs are potential targets where antisense oligonucleotides could exert specific effects. Nevertheless, because of phosphodiester degradation and inhibition of virus entry by phosphorothioate oligonucleotides, the relative roles of specific and non-specific mechanisms by which antisense oligonucleotides inhibit HIV-1 in both acute and chronic infections within cells have been incompletely elucidated. In contrast, inhibition by oligonucleotides of HIV-1 reverse transcriptase-dependent DNA synthesis has been well studied in cell-free systems. Phosphorothioate oligonucleotides are competitive inhibitors of primer binding in a manner largely independent of their nucleotide sequences (19,20). Phosphodiester oligonucleotides inhibit reverse transcription in a sequence-specific manner, indicating that their activity depends on interaction with the viral genome (27,28). In this case, both RNase-H-dependent and independent mechanisms have been demonstrated by the use of oligonucleotides in the α configuration, which do not activate RNase-H (29), and by the use of mutant reverse transcriptase devoid of RNase-H activity (27,28), suggesting that these oligonucleotides may act by physical block of RT fixation or through arrest of reverse transcription.

Concerning messenger RNA, two principal mechanisms have also been reported for the inhibition of protein synthesis in cell-free systems (9,30). Antisense oligonucleotides can block translation by an RNase-H-independent process if they are complementary to the 5′ non coding region of the message or to the splice acceptor site (29,31−34). In contrast, if they are directed against the coding region the inhibition is RNase-H dependent.

The question of whether antiviral activity in cells is RNase-H dependent has not been unambiguously determined. We have investigated the mechanisms of action of oligonucleotides delivered by immunoliposomes on acutely and chronically HIV-infected human T cells. We used nuclease sensitive and resistant oligonucleotides and oligonucleotides that do or do not elicit RNase-H-catalyzed RNA degradation. These oligonucleotides were targeted to the coding region of the *rev* gene and delivered into HIV-infected cells by the use of immunoliposomes.

MATERIALS AND METHODS

Synthesis and purification of phosphorothioate and phosphodiester oligonucleotides

All oligodeoxynucleotide syntheses were performed with automated DNA synthesizers (Applied Biosystems, Foster City, CA, USA, models 380-B or 381A). Unmodified (n-anti-*rev* and n-reversed-*rev*) and phosphorothioate β-oligonucleotides were synthesized by Lynx Therapeutics (Foster City, CA, USA). The phosphorothioate oligonucleotides (S-ODN) were synthesized by the method as reported (35). α-phosphodiester and phosphorothioate oligonucleotides were synthesized as already described (36). Purifications were perfomed by reverse-phase HPLC and homogeneity of the products confirmed by capillary electrophoresis.

Sequence of the oligonucleotides used

Oligonucleotides in β configuration: in this study we used antisense sequences known to have anti-HIV-1 activity (28-mer n- or S-β-anti-*rev*: 5′-TCGTCGCTGTCTCCGCTTCTTCCTG-CCA-3′) (25). To verify the sequence specificity of the antisense oligonucleotides, we have chosen two control oligonucleotide sequences, including a reversed sequence for unmodified normal oligonucleotides (28-mer n-β-reversed-*rev*: 5′-ACCGTCCTTC-TTCGCCTCTGTCGCTGCT-3′) and an antisense sequence to herpes simplex virus (HSV; an irrelevant target with respect to the HIV-1 genome) for phosphorothioate oligonucleotide (21-mer S-β-anti-HSV: 5′-GCCGAGGTCCATGTCGTACGC-3′).

Oligonucleotides in α conformation: we used the same HIV-1 *rev* sequence as described above but in inverse orientation due to the fact that α-oligonucleotides hybridize in parallel orientation, in contrast to β oligonucleotides which hybridize in antiparallel orientation (29,31). In this case the β-antisense sequence is the same as the α-reversed and β-reversed sequence is the same as the α-antisense (28-mer α-anti-*rev*: 5′-ACCGTCCTTCTTCG-CCTCTGTCGCTGCT-3′) and (28-mer α-reversed-*rev*: 5′-TC-GTCGCTGTCTCCGCTTCTTCCTGCCA-3′).

Tm of RNA−oligonucleotide interaction

RNA corresponding to the target sequence 5′-AUGGCAGGA AGAAGCGGAGACAGCGACGAA-3′ (Genset SA, Paris) was incubated with each of the antisense and control oligonucleotides at equimolar (0.5 μM) concentrations. The OD was followed at 260 nm on a Uvikon 940 for temperature increments from ambient to 80°C or 80°C to ambient at 30°C/h.

Antibodies

The purification and use of these mouse IgG2a,k monoclonal antibodies in conjunction with protein A-bearing liposomes has been reported (37). Antibody B1.23.2 is specific for the human major histocompatibility complex (MHC)-encoded HLA-B and C molecules. Antibody H100.5/28, specific for mouse MHC-encoded H2-K molecules not expressed on human cells, is used as a control.

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). C-8166, provided by R.Gallo, is a human T-cell line containing a defective genome of the human T-cell leukemia virus type 1 (HTLV-I) (38). This cell line is known to form syncytia very efficiently in the presence of HIV-1 infected cells (39). HIV-1 (BRU, provided by L.Montagnier) 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.

Preparation of liposomes

Small unilamellar liposomes containing oligonucleotides encapsulated at a concentration of 4−6 mg/ml, were prepared and coupled to Protein A as described (25,40). Liposomes always contained in their aqueous space oligonucleotides at their original concentrations and were diluted in medium to the desired final olignucleotide concentration. This concentration was determined by the use of oligonucleotides that were trace-labeled with

[γ-^{32}P]ATP at the 5′ end by T4 polynucleotide kinase (Amersham). Liposomes containing buffer were diluted to equivalent phospholipid concentrations (41).

Study of inhibition of syncytium formation

The syncytium assay was performed in 96-well or 24-well flat bottom microtitre plates. 5×10^4 HIV-1 chronically infected CEM or uninfected C8166 cells were incubated 1 h on ice, in the presence or absence of 10 μg/ml of antibody and various concentrations of liposomes or with free oligonucleotides, in a total of 100 μl. Then, 5×10^4 CEM/HIV-1 or C8166 (100 μl) were added and incubated at 37°C for 24 h. The number of giant cells in each well was quantified by microscopic examination. Cells were fixed by paraformaldehyde overnight after their incubation for purposes of photography.

Antiviral assay on acutely and chronically infected CEM cells

CEM cells were infected and viral RT and p24 measured as previously reported (25).

RESULTS

Tm of oligonucleotide–RNA interactions

The Tm for the interaction of each oligonucleotide with 30-mer RNA of the target sequence was determined by spectrophotometry. Curves were obtained twice and were reproducible. The Tm for all of the antisense oligonucleotides was comparable (β-n-antisense = 74°C; β-S-antisense = 66.2°C; α-n-antisense = 70.2°C; α-S-antisense = 69.8°C. No transition was seen for any of the control oligonucleotides except for the n-reverse phosphodiester at low temperature (38°C).

Inhibition of syncytium formation

Phosphorothioate oligonucleotides are reported to bind to CD4 molecules and to viral gp120 and to inhibit binding and fusion of HIV-1 with cells (17,18). We have shown that phosphorothioate oligonucleotides encapsulated in liposomes inhibit acute viral infection as efficiently as free oligonucleotides (25). It was therefore important to determine if the action of these liposome-encapsulated oligonucleotides depended in part on their leakage from the liposomes or exocytosis from cells, resulting in inhibition of virus–cell interaction. The ability of oligonucleotides encapsulated in immunoliposomes to inhibit the cell-to-cell transmission of virus by HIV-induced syncytium formation was evaluated in comparison with oligonucleotides free in solution. Uninfected CD4$^+$ C8166 cells were co-cultured with chronically HIV-1 infected CEM cells (expressing gp120 molecules) for 12 or 24 h and the number of syncytia was then determined in the presence or absence of free or liposome encapsulated oligonucleotides.

In its free form, the S-β-anti-*rev* oligonucleotide completely blocked syncytium formation with a concentration resulting in 50% inhibition (IC_{50}) of approximately 0.5 μM (Fig. 1). The same results were obtained with the sequence control S-anti-HSV oligonucleotide (data not shown). The n-β-anti-*rev* oligonucleotide did not inhibit syncytium formation up to a concentration of 25 μM (Fig. 1). No targeted or untargeted liposome-encapsulated oligonucleotides inhibited syncytium formation (Fig. 1) when tested at a final concentration of 0.5 μM, which is the concentration at which they efficiently inhibit HIV-1 replication (25). We determined that all liposome preparations were devoid of cytotoxicity for both infected and uninfected CEM cells as measured by trypan blue exclusion and radiolabeled base incorporation (data not shown). In consequence, the effects previously observed with oligonucleotides in natural configuration delivered by immunoliposomes were due to their action within cells and not on the entry process of HIV-1. Comparison of the activity of free and liposome-encapsulated oligonucleotides thus permits discrimination of extracellular and intracellular inhibition.

Antiviral effects of oligonucleotides free in solution or encapsulated in immunoliposomes on acutely infected cells

We evaluated the effect on HIV-1 replication of free and liposome-encapsulated phosphodiester or phosphorothioate

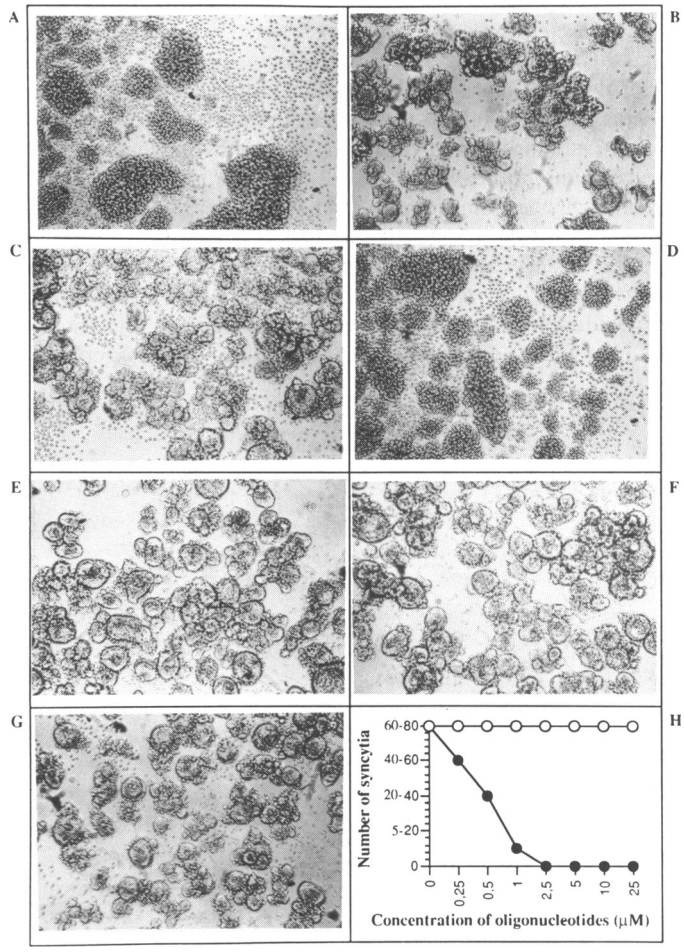

Figure 1. Inhibition of syncytium formation. Uninfected C8166 cells were co-cultured with uninfected or chronically HIV-1 infected CEM cells in the presence or absence of free or liposome encapsulated oligonucleotides. The photographs are of cells fixed 24 h after initiation of culture. (**A**) C8166 and uninfected CEM cells. (**B**) C8166 cells mixed with chronically HIV-1 infected CEM cells. (**C**) C8166 and HIV-1/CEM with 1 μM n-β-anti-*rev* oligonucleotide free in solution. (**D**) with 1 μM S-β-anti-*rev* oligonucleotide free in solution. (**E**) with 0.5 μM (n-β-anti-*rev*)-liposomes targeted to HLA class I molecules. (**F**) with 0.5 μM (S-β-anti-*rev*)-liposomes targeted to HLA class I molecules. (**G**) with (empty)-liposomes targeted to HLA class I molecules. (**H**) Dose–response of free n-β-anti-*rev* (open circles) and S-β-anti-*rev* oligonucleotides (closed circles). The number represents the range of syncytia per Costar well. Similar results were obtained in three independent experiments. The level of expression of p24 in control infected cells was 1800–2200 ng/ml.

oligonucleotides in the α configuration in comparison with the activity of those in β configuration. Results of the antiviral effects of oligonucleotides in their free form on acutely infected cells are presented in Fig. 2. Phosphorothioate oligonucleotides, that are nuclease resistant, inhibited the replication of HIV-1 as measured by p24 production in a manner that was independent of their configuration (α or β). The same results were obtained by measuring reverse transcriptase activity (data not shown). As previously reported for α and β phosphorothioate oligonucleotides, the inhibition was dose-dependent and without sequence specificity (3,10,12,25,42,43). The IC_{50} was about 0.5 μM for all phosphorothioate oligonucleotides. This activity is thought to be due to a direct effect on the HIV entry process as shown in Fig. 1, but an inhibition of RT cannot be formally excluded since these oligonucleotides have been shown to inhibit the action of this enzyme in cell-free systems (19,20). Unmodified oligonucleotides (n-β-anti-*rev;* n-β-reversed-*rev*) tested in medium containing 10% FCS showed no inhibitory effects, probably due to their rapid degradation in culture medium. In contrast, the phosphodiester antisense oligonucleotide in the α configuration, which is relatively insensitive to the degradation by nucleases (44,45), inhibited the replication of HIV-1 ($IC_{50} \leq 1$ μM) in a sequence-specific manner, since the n-α-reversed-*rev* oligonucleotide did not show any activity up to a final concentration of 10 μM. Another difference between α and β oligonucleotides in addition to their sensitivity to nucleases is the inability of the former to induce degradation of RNA by RNase-H (29,31,32). Thus, the action of these oligonucleotides in inhibition of HIV replication does not appear to be RNase-H-dependent. This result differs from those published previously showing that the activity of an α phosphodiester oligonucleotide in acute HIV-1 infection was inefficient and non sequence-specific (43). Differences with our results may be explained by the differences of length of oligonucleotides used (12-mer versus 28), of targeted sequences (splice acceptor site versus region of the initiation codon) and of the cell type used, since these parameters have important consequences for the efficacy of oligonucleotides.

Oligonucleotides were encapsulated in liposomes and their antiviral activity evaluated on acutely HIV-1 infected CEM cells (Figs 3 and 4). Liposomes containing α or β phosphorothioate oligonucleotides (S-β-anti-*rev;* S-β-anti-HSV; S-α-anti-*rev*; S-α-reversed-*rev*) targeted to acutely infected CEM cells by HLA specific antibodies (B1.23.2) inhibited HIV-1 p24 expression to a similar extent at a concentration of 0.5 μM (82, 73, 75 and 75% inhibition, respectively) and RT activity (93, 84, 85 and 84% inhibition, respectively). As for these molecules in their free form, inhibition was not dependent on the oligonucleotide sequence, since antisense and control sequences inhibited viral replication to the same extent. Nevertheless, their efficiency was improved by a factor of five when encapsulated in targeted-liposomes with respect to that observed in their free form. In this case, as demonstrated by an absence of inhibition of viral gp120−cell interaction (Fig. 1), the activity can only be related to the action of phosphorothioate oligonucleotides inside the cells. Thus, phosphorothioate oligonucleotides directly delivered into cells by immunoliposomes acted non-specifically on RT as previously shown in cell-free systems (19,20).

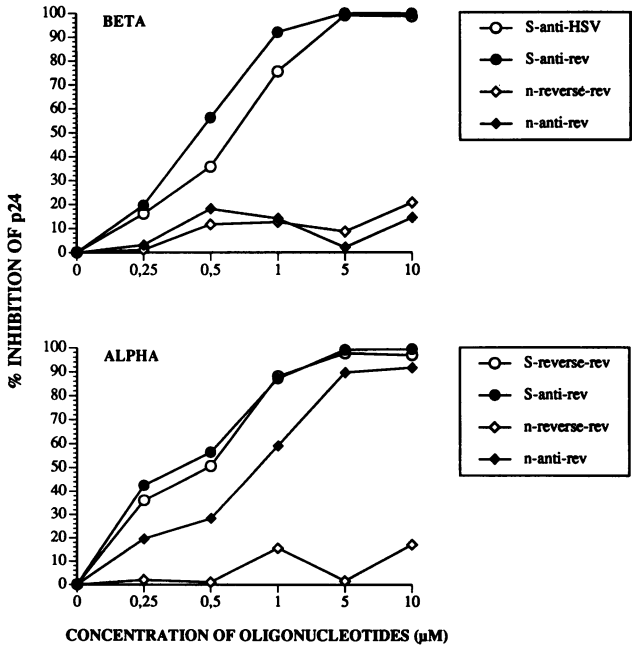

Figure 2. Antiviral effects of oligonucleotides free in solution on acutely infected cells. CEM cells were infected with HIV-1 and subsequently incubated with various concentrations of oligonucleotides. The total incubation time post-infection was 7 days. The p24 expression was determined in the supernatants. Data are given as percent of inhibition of p24 expression, compared with the infected controls. The means of two separate experiments are given. The SD did not exceed 10% of this mean. The level of expression of p24 in control infected cells was 1900−2300 ng/ml.

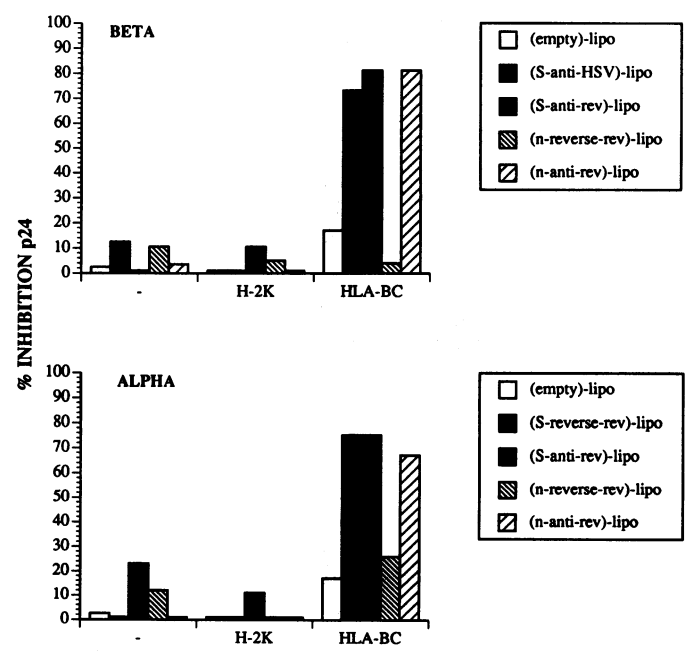

Figure 3. Antiviral effects of oligonucleotides encapsulated in immunoliposomes on acutely infected cells. CEM cells were infected with HIV-1. Oligonucleotides encapsulated in liposomes were subsequently added to some wells, at a final concentration of 0.5 μM, in the presence (as specified), or not (−), of antibodies. The total incubation time post-infection was 7 days. The p24 expression was determined in the supernatants and the data are given as percent of inhibition of p24 expression compared with the infected controls. The means of three separate experiments are given. The SD did not exceed 10% of this mean.

The encapsulated α or β phosphodiester oligonucleotides acted in a sequence specific manner, since the antisense *rev* oligonucleotides were active, but the control sequences n-reversed-*rev* of the same base composition had practically no effect (Figs 3 and 4). Thus, in agreement with previous reports, liposome encapsulation resulted in protection of β unmodified oligonucleotides from nuclease attack and permitted their activity to be demonstrated (22–25). As for the phosphorothioate oligonucleotides, the efficiency of the n-α-anti-*rev* was improved by a factor of five (Fig. 5). The antiviral effects depended on specific cellular delivery of the liposome-encapsulated antisense reagents, as none were seen for targeted empty liposomes or for oligonucleotide-containing liposomes in the absence of antibody or targeted with control antibodies that do not bind to CEM cells (Figs 3 and 4).

The mechanism of action appears not to be dependent on the activity of RNase-H since both α and β phosphodiester oligonucleotides acted to the same extent and confirmed results obtained with α oligonucleotides in their free form. The specificity of action indicates that this effect is due to an interaction with viral genomic RNA prior to RT and integration, or with mRNA newly produced by the integrated virus, or both, but not with RT itself. To evaluate the mechanism of action of oligonucleotides on mRNA newly produced by the integrated virus we studied the activity of these oligonucleotides on chronically HIV-1 infected CEM cells.

Antiviral effects on chronically infected cells of oligonucleotides free in solution or encapsulated in immunoliposomes

We investigated the antiviral activities of free and immunoliposome-encapsulated oligonucleotides on chronically HIV-1 infected CEM cells. Since chronically infected cells have down-modulated their CD4 molecules and HIV replication is RT independent (because the viral genome is integrated) (21), neither of the potential targets for a known non-specific inhibitory mechanism were available. In this model, all phosphodiester oligonucleotides (α and β) were inactive, both in their free form or encapsulated in immunoliposomes. As shown in Figs 6 and 7, only the S-β-anti-*rev* oligonucleotide inhibited viral proliferation in a dose-dependent and sequence-specific manner. The dose necessary to achieve substantial inhibition in its free form was higher than that used for acute infection (IC_{50} about 7.5 μM), in agreement with previous results (16). The inhibition obtained with HLA-targeted liposome-encapsulated S-anti-*rev* was observed at concentrations 40–50 times lower than that required for the free form ($IC_{50} \leq 0.2$ μM). No inhibition was obtained with empty liposomes or with liposomes containing oligonucleotides either in the absence of antibody or in the presence of an irrelevant antibody (data not shown).

In this chronic infection model, S-α-anti-*rev* and S-α-reversed-*rev* free or encapsulated in immunoliposomes, which were active on acutely infected cells, failed to inhibit the replication of HIV. The inefficiency in chronic infection of the n-β-anti-*rev* oligonucleotide in liposomes targeted to MHC class I molecules was probably due to the greater amount of viral mRNA produced by the integrated virus than that present immediately after infection, in association with a greater sensitivity to intracellular nucleases of phosphodiester as compared with phosphorothioate and α phosphodiester oligonucleotides. Nevertheless, even with oligonucleotides resistant to nucleases (n-α-anti-*rev* and S-α-anti-

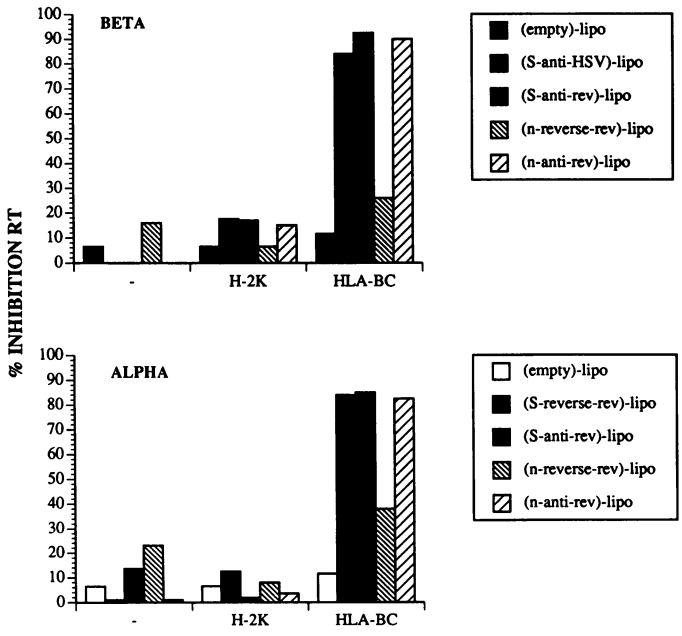

Figure 4. Antiviral effects of oligonucleotides encapsulated in immunoliposomes on acutely infected cells. The details are as reported in Fig. 3. The RT activity was determined in the supernatants and the data are given as percent of inhibition of RT activity compared with the infected controls. The means of three separate experiments are given. The SD did not exceed 15% of this mean.

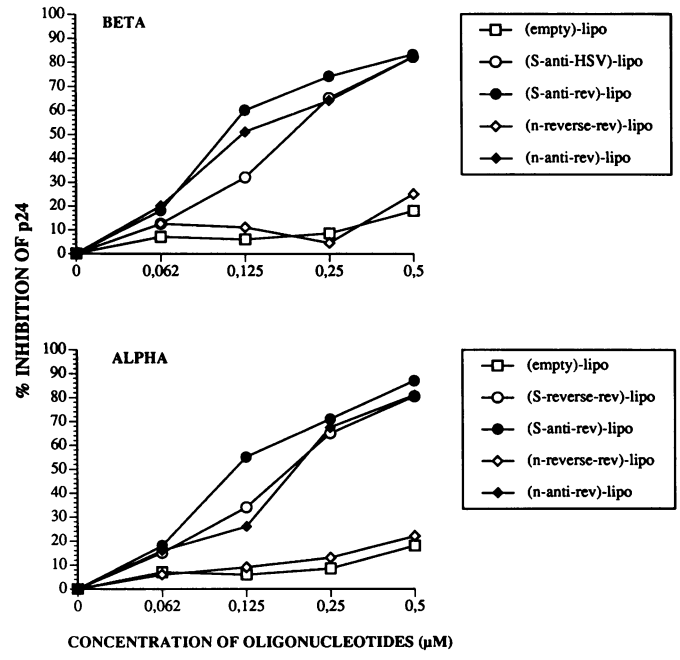

Figure 5. Dose–response curve of antiviral effects of oligonucleotides encapsulated in immunoliposomes on acutely infected cells. Acutely infected CEM cells were incubated alone or with anti-HLA antibody (B.1.23.2) and with various concentrations of oligonucleotides encapsulated in liposomes. At day 7, p24 expression was determined in the supernatants. The data are given as percent of inhibition of p24 expression, compared with the infected controls. The level of expression of p24 in control infected cells was 1900–2300 ng/ml. The means of three experiments are given. The SD did not exceed 10% of this mean.

rev), we did not detect an antiviral effect. Since only the S-β-anti-*rev* oligonucleotide, which is both relatively resistant to degradation and can elicit the degradation of RNA by RNaseH was active, the inefficiency of α-oligonucleotides in immunoliposomes or free in solution suggested that the mechanism of inhibition in chronically infected cells of these oligonucleotides is mediated by an RNase-H dependent process. In chronically infected cells the targets of oligonucleotides are the mRNAs transcribed from integrated proviral DNA. Thus, our results in chronically infected cells agree with those obtained in cell-free systems showing that oligonucleotides targeted to coding regions block protein synthesis primarily by a mechanism dependent on RNase-H activity (29,31–33).

DISCUSSION

The results presented in this paper confirm our earlier report on the anti-HIV-1 activity of liposome-encapsulated oligonucleotides in the β conformation (25). The additional results presented here using oligonucleotides in the α-configuration permit a dissection of the several different mechanisms by which antisense oligonucleotides may inhibit viral replication in acutely and chronically HIV-1-infected cells in tissue culture. The first mechanism is observed for free α and β phosphorothioate oligonucleotides independent of their sequence and is modeled by oligonucleotide-mediated inhibition of syncytium formation between chronically infected cells expressing gp120 and non-infected cells expressing CD4 (Fig. 1). Phosphorothioate oligonucleotides have been previously shown to bind to CD4 and to the V3 loop of viral gp120 (17,18). This mechanism of inhibition of virus entry could be operative *in vitro* in cycles of viral release and re-infection subsequent to the initial infection, since the phosphorothioate oligonucleotides we used are relatively nuclease resistant and are present throughout the culture period. It not clear that similar inhibition of gp120-CD4 mediated viral fusion would occur *in vivo* at achievable oligonucleotide concentrations. This inhibition of syncytium formation was not observed when oligonucleotides were encapsulated in liposomes. Thus, in agreement with previous studies, oligonucleotides do not leak from liposomes (46) or from cells in sufficient quantity to inhibit gp120–CD4 interaction by this mechanism. Moreover, the neutral liposomes used in our studies do not interfere with HIV infectivity, in contrast to some charged liposomes that can modulate the infection of HIV (47,48). Consequently, the effects observed on HIV-1 replication when oligonucleotides were encapsulated in liposomes were due only to oligonucleotides delivered through a system normalized for transport, permitting direct comparison of the activity of different oligonucleotides within cells.

The second mechanism in inhibition of viral production is limited to acute infection and demonstrated by α and β phosphorothioate oligonucleotides independent of their sequence. This mechanism is observed for liposome-encapsulated oligonucleotides as well as free phosphorothioate oligonucleotides, so must take place within cells. It presumably represents either binding to RT or some other non-specific mechanism interfering with reverse transcription.

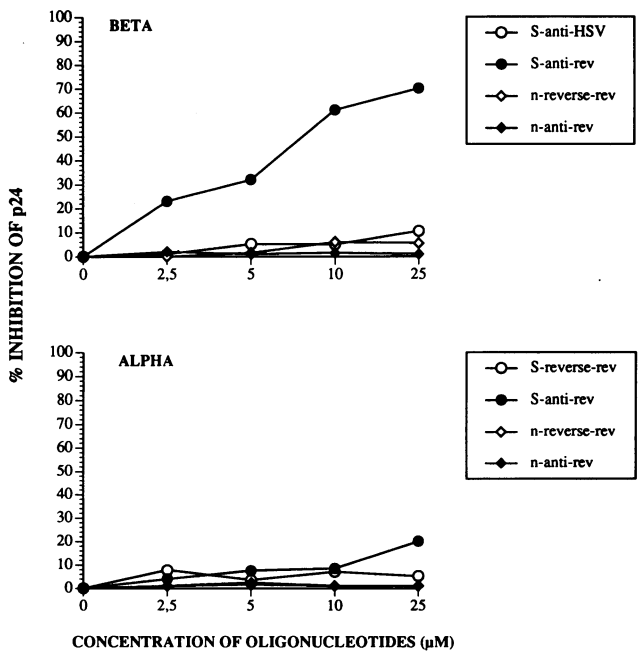

Figure 6. Antiviral effects of oligonucleotides free in solution on chronically infected cells. Chronically infected CEM cells were incubated with various concentrations of oligonucleotides. At day 4, p24 expression was determined in the supernatants. The data are given as percent of inhibition of p24 expression, compared with the infected controls. The level of expression of p24 in control infected cells was 2000–2400 ng/ml. The means of four separate experiments are given. The SD did not exceed 10% of this mean.

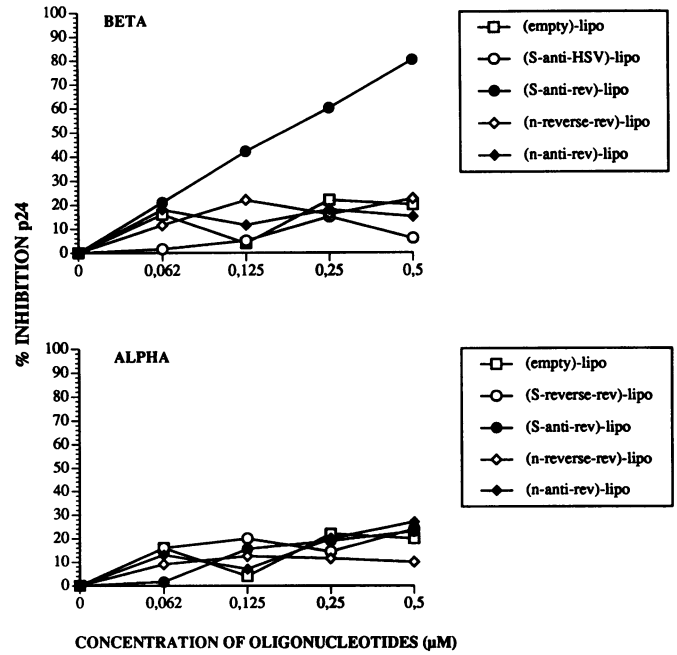

Figure 7. Antiviral effects on chronically infected cells of oligonucleotides encapsulated in immunoliposomes. Chronically infected CEM cells were incubated alone or with anti-HLA antibody (B.1.23.2) and with various concentrations of oligonucleotides encapsulated in liposomes. At day 4, p24 expression was determined in the supernatants. The data are given as percent of inhibition of p24 expression, compared with the infected controls. The means of two separate experiments are given. The SD did not exceed 10% of this mean.

The third mechanism is sequence specific and is defined by the inhibition of acute infection by free α or liposome-encapsulated α and β phosphodiester oligonucleotides. The inability of free β oligonucleotides to act is explained by their rapid degradation in tissue culture media. The mechanism of viral inhibition appears to be RNase-H independent, in that both α and β oligonucleotides were active. α- and β-oligonucleotides of equivalent length and complementary to the same target had comparable affinity for their target sequences, as shown by UV melting and thermal elution experiments (34, 43), and this was confirmed for the sequences used here. The fact that the α antisense oligonucleotide, which is much more nuclease resistant, did not have greater activity than the β oligonucleotide is consistent with three possible interpretations: (i) nuclease activity in the cytoplasm is modest; (ii) the molecules are present in the cytoplasm for a only short period of time before their diffusion to binding sites in the nucleus (49); (iii) the inability of α-oligonucleotides to activate RNase-H, as compared with the β-oligonucleotides, might be compensated by greater nuclease resistance.

Oligonucleotides encapsulated in liposomes pass first into the cytoplasm and then rapidly diffuse into the nucleus (23). That the association of oligonucleotides with liposomes, which is known to prolong their release from endocytic vesicles into the cytoplasm, increased their activity only by a factor of five is consistent with this observation, since in acute infection the site of action of oligonucleotides is primarily on RT, which takes place in the cytoplasm. In contrast, the liposome-encapsulated β phosphorothioate antisense oligonucleotide was 50 times more active than the free form in chronic infection, as the site of action in this case is expected to be primarily in the nucleus.

In cell-free systems two studies using purified RT have indicated that oligonucleotides targeting regions near the primer binding site can inhibit RT by RNase-H-independent mechanism(s) after specific binding on the template (27,28). One report using RTs from avian myeloblastosis and murine leukemia viruses on a β-globin mRNA template suggest that oligonucleotides directed to coding regions not adjacent to the primer do not act by this mechanism but needed rather to be substrates for RNase-H (28). In our experiments, RNase-H-dependence seems not to be necessary, since an α oligonucleotide complementary to the initiation codon region of *rev*, which is much further from the primer binding site, blocked the replication of HIV in the acute infection model. It should be pointed out that the α-oligonucleotide-mediated antiviral activity observed in cells could still be RNase-H dependent, in the sense that a physical block of RT polymerization by fixation of RNase-H to the duplex antisense oligonucleotide–viral RNA may not necessarily depend on the nuclease activity of RNase-H (50).

The fourth mechanism is seen for chronically infected cells and depends both on the nuclease resistance and RNase-H activating properties of a sequence-specific S-β-oligonucleotide. These properties are consistent with degradation of *rev* mRNA by RNase-H and agree with numerous experiments in cell-free systems showing that an RNase-H-independent mechanism exists predominantly if oligonucleotides are directed to 5′ non-coding region or to the splice acceptor site (29,31–34,51–53). In contrast, if the oligonucleotides are complementary to the coding region, RNase-H activity is normally necessary for antisense effects (31–34). The results presented here confirm these studies and are consistent with those reported for an α-oligonucleotide directed against the coding region of the N-protein of VSV in cells (54). However, in preliminary results previously reported by some of us, an α-phosphorothioate oligonucleotide corresponding to the same *rev* sequence as used here inhibited the replication of HIV in chronically infected H9 cells, suggesting an RNase-H-independent mechanism (42). Our negative results for chronic infection using both phosphodiester and phosphorothioate α-oligonucleotides that were active in inhibition of acute infection were repeated several times with different lots of oligonucleotides. Further, the encapsulation in liposomes that augmented the activity of the β-phosphorothioate antisense oligonucleotide by a factor of 50 had no effect on the activity of α-oligonucleotides in our experiments.

The apparent requirement for the use of modified oligonucleotides in chronic infection raises the concern of their possible toxicity for *in vivo* use. The demonstration here of equivalent activity of liposome-encapsulated oligonucleotides at lower doses than is necessary when oligonucleotides are used in their free form is promising in this context.

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