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Antiviral Research 33 (1996) 11–20

Antiviral Research

Inhibition of HIV replication by immunoliposomal antisense oligonucleotide

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Received 24 November 1995; accepted 12 July 1996

Abstract

The sequence-specific suppression of HIV-1 replication using CD4 monoclonal-antibody-targeted liposomes, containing Rev antisense phosphorothioate oligonucleotides is described. Liposomes were prepared by encapsulating the 20-mer antisense DNA sequence of the *rev* HIV-1 regulatory gene, in the form of a phosphorothioate oligonucleotide. Specific targeting was accomplished by conjugating anti-CD4 mouse monoclonal antibody to the surface of the liposomes. HIV-1-infected H9 cells as well as peripheral blood T-lymphocytes were incubated with the immunoliposomes of antisense found to have potential antiviral effect. HIV-1 replication was reduced by 85% in antisense immunoliposome-treated H9 cells and peripheral blood lymphocytes, whereas the inhibition of HIV-1 replication was not observed using either empty immunoliposomes or immunoliposomes containing scrambled Rev phosphorothioate oligonucleotide sequences. The antiviral activity of both the free and the encapsulated oligonucleotides were assessed by p24, reverse transcriptase (RT) assays and polymerase chain reaction (PCR) analysis. Liposome preparations demonstrated minimal toxicity in H9 as well as in peripheral blood lymphocyte cell culture experiments. These in vitro culture results demonstrate the potential efficacy of immunoliposomes to inhibit HIV replication.

Keywords: Phosphorothioate; Rev; Immunoliposome; Polymerase chain reaction; Antisense; Human immunodeficiency virus

1. Introduction

Antisense oligonucleotides have been used in a number of studies as effective inhibitors of virus replication (Ropert et al., 1994; Li et al., 1993).

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PII S0166-3542(96)00993-X

The emergence of the AIDS epidemic and the need for different approaches to control retroviruses have led to numerous studies in the use of antisense oligonucleotides as inhibitors of human immunodeficiency virus (HIV) replication. The antiviral efficacy of both unmodified and modified antisense oligonucleotides has been evaluated (Li et al., 1993; Zelphati et al., 1994a,b). Successful results have been reported with oligonucleotides targeted to different sites of viral RNA or mRNA. Antisense technology offers a possible method for studying the roles of individual proteins in viable cells and may have therapeutic potential. However, the native oligonucleotides are highly susceptible to nuclease degradation (Akhtar et al., 1991; Shaw et al., 1991), and their uptake by cells is inefficient (Loke et al., 1989; Yakubov et al., 1989).

An approach to overcome these problems has been to bind modified oligonucleotides (phosphorothioates) to phospholipids (Shea et al., 1990) or other hydrophobic residues (Saison-Behmoaras et al., 1991), including cholesterol (Kreig et al., 1993). Cholesterol modification has been shown to increase the potency of the antisense oligonucleotides (Kreig et al., 1993; Boutorine and Kostina, 1993). We describe here an alternative strategy to augment the association between the oligonucleotide and infected cell, enhance the oligonucleotide entry into the cell and protect the oligonucleotide from degradation.

In this study we report the synthesis of phosphorothioate antisense oligonucleotide and its encapsulation in liposomes. We tagged the liposomes with anti-CD4 monoclonal antibody, which allows them to be targeted to a specific cell population, since HIV predominantly attacks cells that bear CD4 receptors (Dalgleish et al., 1984). We have previously shown that liposomes can be used for intracellular delivery of the anti-cancer drug, doxorubicin, in the treatment of breast cancer, by targeting specific tumor antigens (Rahman et al., 1989). We demonstrate here the use of antibody-targeted liposomes for the intracellular delivery of phosphorothioate antisense complementary to the HIV *rev* region.

2. Materials and methods

Anti-CD4 mouse monoclonal antibody (IgG) was purchased from Biosource International, Camarillo, CA. All lipid components for the liposome preparation and azidothymidine (AZT) were obtained from Sigma Chemical (St. Louis, MO). [methyl-^{3}H]Deoxythymidine triphosphate ([^{3}H]TTP), specific activity 10–25 Ci/mmol, was purchased from DuPont-NEN (Boston, MA). Poly(rA)·oligo(dT) 12–18 and poly(dA)·oligo(dT) 12–18 primers were obtained from Pharmacia/P-L Biochemical (Piscataway, NJ). Glass fiber filters and nitrocellulose were purchased from Millipore (Bedford, MA). Triton X-100, tRNA and all other chemicals were obtained from Sigma. Polymerase chain reaction (PCR) kits and probes were purchased from Perkin Elmer Cetus (Norfolk, CT).

2.1. Cell lines and HIV infection

Peripheral blood lymphocytes (PBL) were purified from peripheral blood mononuclear cells of donors, seronegative for HIV and hepatitis B infection, after leukophoresis and purified by countercurrent centrifugal elutriation (Wahl and Smith, 1991). PBLs were activated with phytohemagglutinin (PHA) (10 μg/ml final concentration) for 48 h in the presence of polybrene (2 μg/ml) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FCS), glutamine, antibiotics and 10% interleukin-2 (IL-2) (50 half-maximal units/ml; Advanced Biotechnologies, Columbia, MD). Cells were exposed to HIV-1 (HTLV-III$_B$) at a multiplicity of infection (MOI) of 0.01 infectious virus per target cell (Hansen et al., 1992). After the 2-h viral adsorption interval, cultures were washed and re-fed with fresh medium. Culture medium was half exchanged with fresh medium every 2–3 days. H9 cells are a transformed line of human lymphocytes permissive for HIV-1. Cultures were maintained using RPMI-1640 supplemented with 10% FBS, 2% L-glutamine and 1% penicillin–streptomycin and the infection was carried out in the same way as in PBL.

Each well of a six-well plate received 10^5 cells in 5 ml of medium. Control and infected cells were cultured with the different formulations of oligonucleotides or AZT at specified concentrations for 7 days. The supernatants and cell pellets were then harvested and examined for virus growth, as described below. Cell viability was determined in all treatments using [^{3}H]thymidine incorporation and trypan blue exclusion assay in comparison with control cultures (Selvam et al., 1993a). The inhibitory properties of the antisense encapsulated immunoliposome on viral replication in H9 cells and PBLs was compared with its inhibitory properties in U937 and HUT-78 cells. U937 is a transformed monocytic cell line and HUT-78 is a transformed lymphocytic cell line from the continuous culture. All these infected cell lines were incubated with the same concentrations of reagent formulations for the same periods of time as previously described (Selvam et al., 1993a).

2.2. Synthesis and purification of oligonucleotides

The oligonucleotide (Compound 5 from Table 1) 5′-CGC TGT CTC CGC TTC TTC CT-3′, a 20-mer, complementary to the 5′-end sequence of HIV-1 Rev mRNA used in this study, was selected after the preliminary screening of 10 different oligonucleotides ranging from 14 to 20 mers at the same region. The oligonucleotide 5′-GTC CGC CTC TCG CTT CTT CT-3′ has a scrambled Rev antisense nucleotide composition and serves as a control. Phosphorothioate oligonucleotides were synthesized on an automated DNA Synthesizer (Model 381 A, Applied Biosystem, Foster City, CA), using phosphoramidite chemistry.

Table 1
Inhibition of HIV-1 expression by antisense DNA oligonucleotides

Compound	Concentration (μg/ml)	% Inhibition	
		p24	RT
1 (15 mer)	1.25	18	12
2 (15 mer)	1.25	5	8
3 (14 mer)	1.25	22	18
4 (20 mer)	1.25	15	10
5 (20 mer)	**1.25**	**46**	**38**
6 (16 mer)	1.25	18	24
7 (15 mer)	1.25	8	10
8 (20 mer)	1.25	12	10
9 (15 mer)	1.25	26	22
10 (20 mer)	1.25	24	28

Synthesis was carried out on a 1-μM scale (74 steps) on controlled pore glass support columns. Deprotection of the oligonucleotides was carried out with concentrated ammonia and triethylamine in a 56°C water bath for 8 h. The DNA was precipitated with cold butanol and the precipitate was dried in a speed vacuum. The oligonucleotides were purified by reverse-phase fast protein liquid chromatography (FPLC system, Pharmacia, Uppsala, Sweden) on a Prep. RPC HR 5/5 reverse-phase column (Pharmacia, Uppsala, Sweden) and eluted by an acetonitrile gradient in 0.1 M triethylammonium acetate buffer. Finally, the oligonucleotide was characterized by polyacrylamide gel electrophoresis (PAGE). The lyophilized powder was suspended in a sterile PBS (pH 7.4), and passed through a 0.2-μm Millipore syringe filter. The oligonucleotides were quantitated by UV absorbance at 260 nm (1 OD $\sim$ 30 μg of DNA).

2.3. Preparation of palmitoylated IgG and immunoliposomes

Mouse anti-CD4 monoclonal antibody IgG (Biosource International) was palmitoylated using *N*-hydroxy succinimide ester of palmitic acid (NHSP) (Sigma, St. Louis, MO). Briefly, 1 mg of antibody was dissolved in 50 mM sodium phosphate buffer (pH 7.8), containing 0.85% NaCl and 0.15% sodium deoxycholate. NHSP was added at a molar ratio of 1:10. The mixture was incubated at 30°C for 6–10 h and then applied to a PD-10 column (Pharmacia-LKB), which was pre-equilibrated with PBS, 0.15% deoxycholic acid (DOC) and eluted with the same. The fatty-acyl antibody eluted in the void volume fraction was pooled and concentrated by ultrafiltration. Control mouse IgG was also coupled to the palmitic acid in the same manner. To assess the attachment of palmitic acid to the IgG molecule, ^{14}C-labeled

palmitic acid was used in some experiments to synthesize labeled NHSP. The concentrated palmitoyl–antibody was then dialyzed against PBS with 0.15% DOC and stored at −20°C in the presence of 0.02% NaN_3.

Sterile pH-sensitive liposome was prepared in aqueous phase composed of 35 mol% cholesterol (Sigma), 45 mol% dioleyl phosphatidyl ethanolamine (DOPE), 10 mol% palmiotyl–CD4 IgG and 10 mol% oleic acid as described earlier (Rahman et al., 1989), with modifications. The final aqueous phase unilamellar liposome containing oligonucleotides had a concentration of 4–6 mg/ml. The pH of the final formulation of either empty liposome or encapsulated liposome with CD4 IgG was always adjusted to pH 8.0. Liposome was sterilized by filtration through 0.45 μm Millipore filters. Size determination performed for liposome of similar composition shows liposome formed by this technique to be primarily unilamellar. Liposome was always contained in its aqueous phase and was diluted in the medium to the desired final oligonucleotide concentration.

2.4. Detection of HIV-1 p24 antigen and reverse transcriptase assay

The presence of p24 antigen was detected and quantitated, using the HIV-1 p24 core profile enzyme-linked immunosorbent assay (ELISA), as described earlier (Selvam et al., 1993a). Briefly, standards from 12.5 to 200 pg/ml were used, and the antigen–antibody complex was probed with a streptavidin–horseradish peroxidase conjugate. The end product is quantitated by the intensity of the yellow color which is directly proportional to the amount of HIV-1 p24 core antigen captured. Color development is read at 492 nm, using a microplate ELISA Reader (BioRad, Richmond, CA).

Reverse transcriptase (RT) enzyme activity of the supernatant and cell extract of the HIV-infected H9 cells was assayed as described earlier (Selvam et al., 1993a). The cell cultures were harvested after 7 days and separated from their supernatants by centrifugation. The cell pellet was solubilized by vigorous vortexing in a lysis buffer, containing 0.2% Triton X-100 in 25 mM Tris–HCL (pH 7.4). An aliquot of 10 μl of the solubilized sample was mixed with 90 μl of the reaction mixture (1.0 M Tris (pH 7.8), 0.2 M DTT/0.2 M $MgCl_2$/ddH_20/universal buffer/[^{3}H]dTTP/poly(rA)·oligo(dT)/poly(dA)·oligo(dT)], vortexed, and incubated at 37°C for 1 h. The reaction was terminated by placing the samples in an ice bath and adding 10 μl of cold tRNA to each tube, followed by 1 ml of ice-cold trichloroacetic acid. The precipitated DNA was harvested on a glass fiber filter (GF/C), using the Millipore Sampling Manifold (Millipore, Bedford, MA) attached to a vacuum source. The incorporation of [^{3}H]thymidine was measured as previously described (Selvam et al., 1993a).

2.5. PCR analysis of HIV-infected H9 cells

The presence of HIV-1 antigens in H9 cells, with and without antisense treatment was determined at the protein level by Western blot (Selvam et al., 1993b) and, at the proviral DNA level, by PCR analysis (Selvam et al., 1993a) as described.

3. Results

3.1. Characterization of liposomes

Encapsulation of antisense oligonucleotides varies with the quality of the lipid components used, since this determines the number of liposomes formed (Zelphati et al., 1994a). The 20-mer Rev or the scrambled sequence of Rev phosphorothioate oligonucleotide was encapsulated into a unilamellar antibody-targeted liposome. In this preparation, DOPE, which enhances the encapsulation efficiency of the antisense oligonucleotide, was the main lipid component.

The number of molecules encapsulated per liposome depends on their solubility in the aqueous phase. In this preparation, we obtained between 75 and 100 oligonucleotide molecules per liposome based on the calculation of 6.5×10^{10} liposomes of (220 ± 55 nm diameter) per μmol of DOPE. Since the antibody is coupled with the lipid component as palmitoyl–IgG and used at

the same concentration for all the formulations, the number of molecules of IgG (anti-CD4 or normal mouse IgG) per liposome was kept constant. Liposome-encapsulated antisense was separated from the free antisense by dialyzing at 4°C against a determined volume of PBS. As the experiments were done with fresh liposome preparations, we did not evaluate the activity and the cytotoxicity of the preparation after the storage.

3.2. Dose response and cytotoxicity

The effect of free and liposomal formulations of antisense on HIV-1 replication was tested in in vitro cultures of peripheral blood T-lymphocytes as well as in the transformed human T-lymphocytes (H9) infected with HIV-1-III$_B$. Results of the dose response in terms of (A) RT and (B) p24 inhibition are represented in Fig. 1. There are eight different formulations with several control reagents to study the non-specific effect. The results in Fig. 1 represent the mean value for fresh PBLs. The 50% antivirally effective dose (EC_{50}) of Rev antisense liposome targeted with anti-CD4 IgG was evaluated for acutely HIV-infected fresh peripheral blood T-lymphocytes. The same was performed for HIV-infected H9 cells also (data not shown). The (EC_{50}) value of this formulation for PBLs is 1.25 μg/ml in terms of RT inhibition and 1.20 μg/ml for p24 inhibition. All possible controls, like empty immunoliposome, scrambled Rev, free and liposomal preparations had non-specific inhibition of less than 5%. According to our experiments, the inhibitory activity with only anti-CD4 IgG-tagged empty liposome was less than 5% and, by mixing free antisense (4 μg/ml) and empty liposome, we had an inhibition of 35%, which was comparatively similar to that of free antisense itself (4 μg/ml) ($\sim$ 38%).

The cytotoxic effects of Rev antisense immunoliposomes at various concentrations have been evaluated in uninfected and acutely HIV-1-infected fresh PBLs for 7 days. Fig. 2 shows that, up to 4 μg/ml concentration of immunoliposomal antisense, there is no toxicity. Although the cytotoxicity experiments were conducted for all eight different formulations of antisense, the toxicity experiment results were shown only for the immunoliposomal antisense, since there is virtually no cytotoxicity with all these formulations. Cell viability was determined by [^{3}H]thymidine incorporation as well as by trypan blue exclusion assays. From the results, it was observed that all these liposome preparations were devoid of cytotoxicity in uninfected as well as in HIV-1-infected PBLs. For comparative purposes, the same experiment was performed in the H9 cell system too, and had similar results (data not shown).

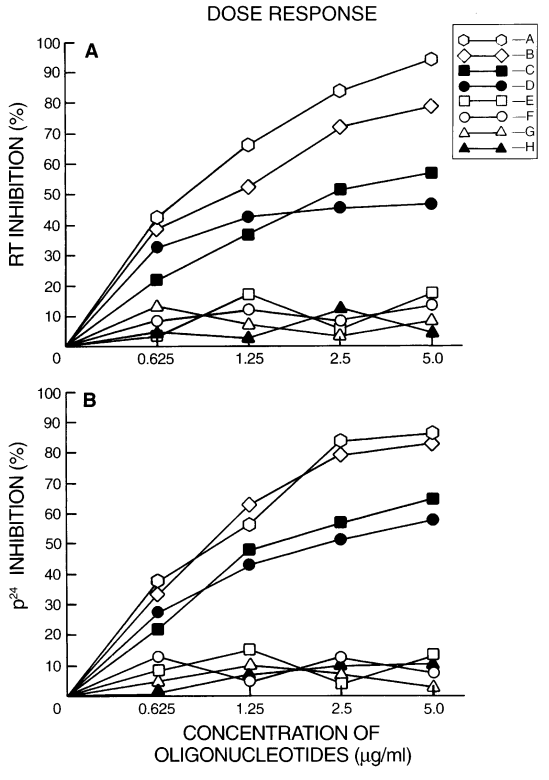

Fig. 1. Dose–response curve of antiviral effects of liposomal and free antisense oligos. HIV-1-infected PBL cells were incubated with different preparations of antisense oligonucleotides in the culture at μg/ml levels. On day 7, (A) RT enzyme activity and (B) p24 expression were determined from the culture supernatants and the results were presented as % inhibition compared with the infected controls as 0%. The mean of the three experiments is presented in the figure. (A) AZT free; (B) liposomal Rev antisense with anti-CD4 IgG; (C) free Rev antisense; (D) liposomal Rev antisense with normal mouse IgG; (E) empty liposomes with anti-CD4 IgG; (F) empty liposomes with normal mouse IgG; (G) free scrambled Rev antisense DNA; (H) liposomal scrambled Rev antisense with anti-CD4 IgG.

3.3. Antiviral effects of oligonucleotides on acutely infected PBLs and H9 cells

Results of the antiviral effects on de novo infected PBLs and H9 cells are presented in Figs. 3 and 4. Oligonucleotides encapsulated in liposome targeted to CD4 molecules by specific mouse monoclonal antibody, strongly blocked (at 4 μg/ml of Rev immunoliposome) the expression of the viral protein p24 and the activity of RT, to the level of 85% in both PBL and H9 cells (Figs. 3 and 4, column 8). This inhibition was sequence specific, since the antisense Rev oligonucleotides were active and, at the same concentration, both free and liposomal scrambled sequence of the same composition had a minimal non-specific effect (Figs. 3 and 4, columns 3 and 5). Both formulations were similar since they were targeted to CD4 molecules. In contrast, free Rev antisense, without encapsulation, has an HIV-inhibitory effect of 30% in the PBL system and 40–45% in H9 cells. The inhibitory activity with only CD4-tagged empty liposome was less than 5% and, by mixing free antisense plus empty liposome, the inhibition was 32% in the PBL system and 35% in H9 cells.

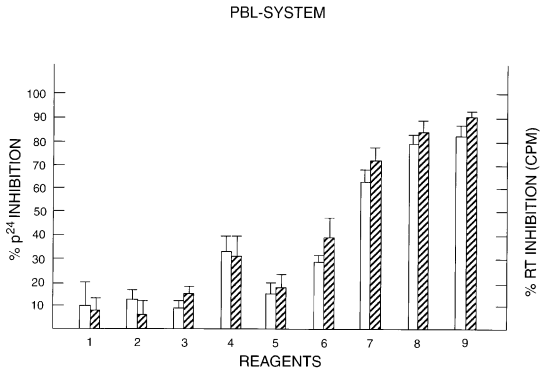

Fig. 3. Inhibition of p24 and RT by free and immunoliposomal antisense DNA in HIV-1-infected PBL cells. All the drug preparations were added at a final concentration as specified. p24 (open column) and RT (grid) enzyme activity was assayed by the standardized procedure and each column represents individual formulation, μg/ml or μl/ml. Results show the mean value (% inhibition) calculated from triplicates, compared with the infected control (0%). Error bar indicates S.D. (1) Empty liposome with normal mouse IgG (20 μl); (2) empty liposomes with anti-CD4 IgG (20 μl); (3) free scrambled Rev antisense DNA (4.0); (4) free Rev antisense (4.0); (5) liposomal scrambled Rev antisense with anti-CD4 IgG (4.0); (6) liposomal Rev antisense with anti-CD4 IgG (1.0); (7) liposomal Rev antisense with anti-CD4 IgG (2.0); (8) liposomal Rev antisense with anti-CD4 IgG (4.0); (9) AZT (5.0).

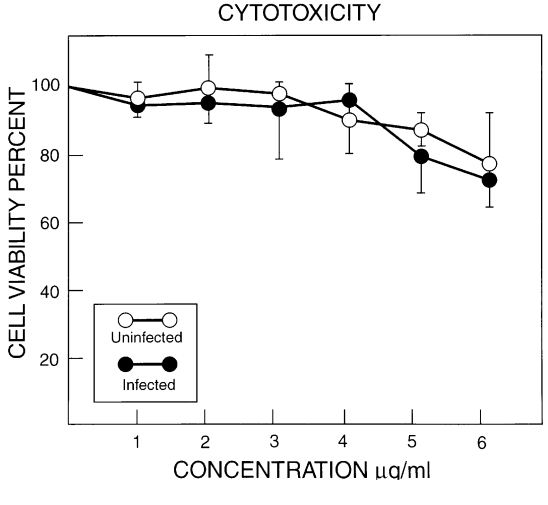

Fig. 2. Cytotoxicity of antisense immunoliposome in PBL cells. Control or HIV-1-infected peripheral T-lymphocytes were incubated in the presence of Rev antisense immunoliposome (1–6 μg/ml). Cell viability was determined in triplicate by [^{3}H]thymidine incorporation assay. Results were expressed as mean of control values (%). Error bar indicates S.D.

3.4. PCR analysis

For additional confirmation of the results, obtained by the treatment of acutely HIV-infected cell cultures with free and immunoliposomal antisense oligonucleotides, we analyzed the viral DNA of cell extracts from each category of treatment by PCR using SK-38 and SK-39 primers in the *gag* gene sequence. Fig. 5 shows that lane 1, which expresses the amplified DNA product (AP) from HIV-1-infected H9 cells without any treatment, binds with ^{32}P-labeled *gag*-specific SK-19 probe. After the treatment with immunoliposomal Rev antisense or AZT, there was no binding with the labeled probe, demonstrating the complete inhibition of viral replication at the proviral DNA level (lanes 7–9 and 11). The treatment with either empty liposome or scrambled Rev antisense liposome does not have any appreciable effect on

HIV replication. Indirect immunofluorescence assay using anti-gp120 and Western blot analysis of

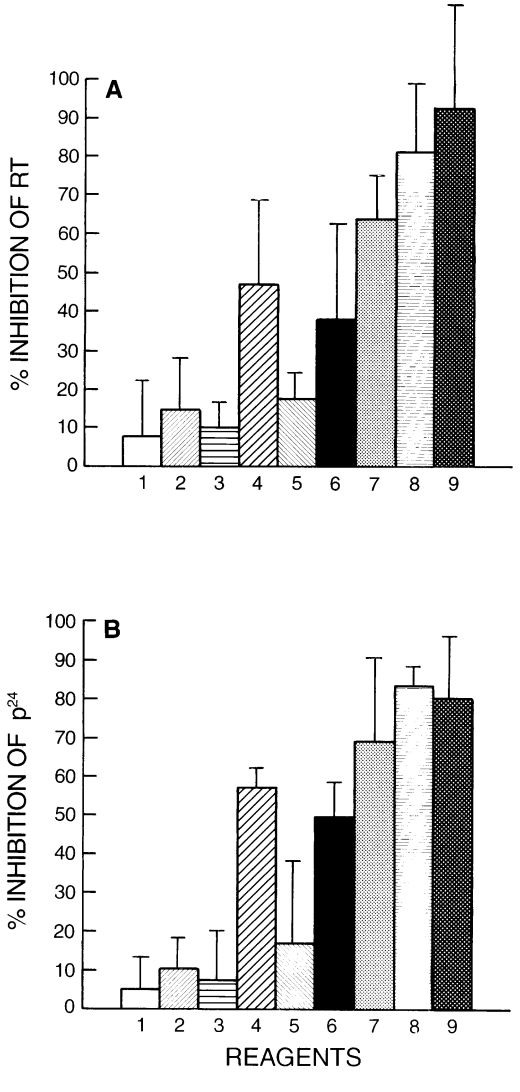

Fig. 4. HIV-1 inhibition in H9 cells by immunoliposomes: all the experimental conditions are same as in Fig. 3, except for the cell line, which consists of transformed human T-lymphocytes. (A) RT inhibition (%); (B) p24 inhibition (%); (1) empty liposome with normal mouse IgG (20 μl); (2) empty liposomes with anti-CD4 IgG (20 μl); (3) free scrambled Rev antisense DNA (4.0); (4) free Rev antisense (4.0); (5) liposomal scrambled Rev antisense with anti-CD4 IgG (4.0); (6) liposomal Rev antisense with anti-CD4 IgG (1.0); (7) liposomal Rev antisense with anti-CD4 IgG (2.0); (8) liposomal Rev antisense with anti-CD4 IgG (4.0); (9) AZT (5.0).

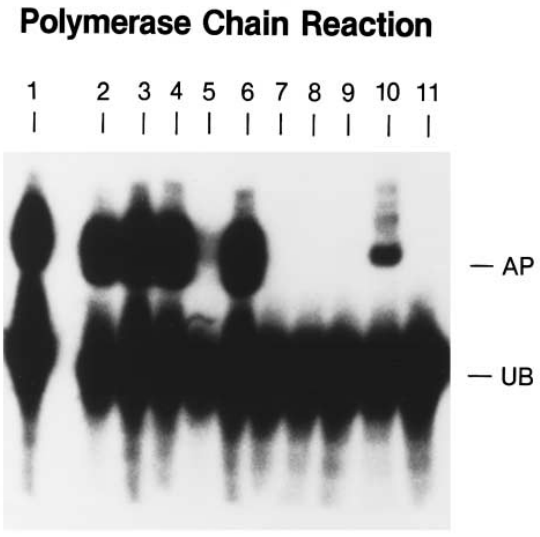

Fig. 5. PCR-electrophoretic analysis of HIV-1 proviral DNA in H9 cells. HIV-1 proviral DNA of the cellular extract was amplified by PCR as described in the text and hybridized with ^{32}P-end-labeled SK-19 probe. Each lane represents the specific preparation and drug treatment concentration in μg/ml of culture. AP, amplified DNA product; UB, unbound radioactive material. (1) HIV-infected H9 cells; (2) empty liposome with normal mouse IgG (20 μl); (3) empty liposomes with anti-CD4 IgG (20 μl); (4) free scrambled Rev antisense DNA (4.0); (5) free Rev antisense (4.0); (6) liposomal scrambled Rev antisense with anti-CD4 IgG (4.0); (7) liposomal Rev antisense with anti-CD4 IgG (2.0); (8) liposomal Rev antisense with anti-CD4 IgG (4.0); (9) liposomal Rev antisense with anti-CD4 IgG (6.0); (10) kit positive control (HIV-1); (11) free AZT (5.0).

HIV proteins were also performed to corroborate the PCR results (data not shown).

4. Discussion

In the present study, these results demonstrate that the replication of the HTLV-III$_B$ strain of HIV-1 in cell culture can be effectively inhibited with immunoliposome-encapsulated antisense oligonucleotides directed against the critical regulatory *rev* gene. The novel aspect of this project is to bypass problems of poor cell permeability and extracellular nuclease degradation, especially by targeting a specific cell population with CD4 receptors, which will be more advantageous in in

vivo clinical trials. Immunoliposomes could serve as a useful model system to study the permeation of oligonucleotides across biological membranes with ^{32}P-labeled phosphorothioate oligonucleotide (Akhtar et al., 1991).

As mentioned earlier, this particular antisense DNA oligonucleotide was selected from the preliminary screening of several oligos (Table 1) in the HIV-1 *rev* gene sequence. In order to assess the delivery by liposome, the oligo was labeled with 5′-^{32}P and incubated with H9/PBL cells both in free as well as liposomal form of equal CPM. After 24 h incubation, the cells were washed twice with PBS and lysed with the lysis buffer. From the result, it was calculated that with the antisense immunoliposome, the intake was 3.8-times greater than that of free antisense. From the preliminary experiments, it was calculated to achieve a maximum of 50% encapsulation of oligos in liposomes. The concentration of the oligo was always determined at 260 nm in the ultraviolet spectrophotometer and it was calculated to have equal concentration in free as well as liposomal forms in all the experiments, by making proper dilutions from the working stocks.

Studies of the mechanism and efficacy of the antisense oligonucleotides in inhibiting viral replication can be approached effectively in an in vitro system, using freshly prepared peripheral blood T-lymphocyte culture, which resembles the process of HIV-1 infection in vivo. Immunoliposomes show the efflux rates from 4 to 7 days, which indicates that the diffusion of most types of oligonucleotides across the membrane is extremely slow and that other mechanisms must account for the cellular uptake of oligonucleotides.

These drug formulations were not cytotoxic at the optimal concentrations needed to protect H9 and PBL cells from HIV infection. At optimal doses, immunoliposomal Rev antisense oligonucleotide blocked 85% of HIV-1 infection without rendering cytotoxicity, when added to either H9 or PBL cells after the adsorption of the virus. Proviral DNA was not detected in these cells by PCR analysis (Fig. 5), which suggests that these agents interfere at early events in the virus life cycle. A study of the cellular uptake of phosphorothioate DNA oligonucleotides showed that they were very efficient inhibitors at low concentrations, suggesting a saturable process (Iverson et al., 1992). Inside the cell, they reached a plateau after 45–60 min, indicating that either the efflux pathways exist or the uptake is saturable (Iverson et al., 1992, 1994). Intracellular uptake and stability studies were performed according to Ropert et al. (1994) and clearly show by autoradiogram (data not shown) that, after 24 h, the oligonucleotide delivered by liposome seems to be intact, whereas the free oligo has degraded faster.

The *rev* gene mediates the export of nuclear-entrapped viral structural mRNA to the cytoplasm and is the preferred target for anti-HIV therapy, because it is unique to HIV and unlikely to regulate host cell functions. Cellular uptake of antisense Rev can inhibit gene expression. Uptake is primarily size dependent, saturable and compatible with the characteristics of receptor-mediated endocytosis. Whether the olignucleotides are used in their free form or in association with a delivery system, their internalization involves either receptor-mediated or fluid phase endocytosis. The movement of the oligonucleotides from the endocytic compartment to the cytoplasmic intracellular targets, or to the nucleus, is problematic.

Although the liposomes are diluted in the medium when added to the cell culture, the oligonucleotides contained within the liposome were able to accumulate at the cell surface in the presence of the appropriate ligand. The potency of these oligonucleotides depends upon the nature of the cell surface molecules to which they bind. Liposomes bound to major histocompatibility complex (MHC)-encoded class I target molecules on L-cells are internalized through non-coated pits (Machy et al., 1987a,b).

A slight increase in HIV infection in control empty liposome-treated H9 cell cultures was observed throughout the experiment and has been corroborated by other investigators (Konopka et al., 1990). The basic tendency of these liposomes is to accumulate in the liver and spleen. This phenomenon can be significantly modified by use of antibody-targeting methods. In particular, these pH-sensitive fusogenic liposomes may prove extremely valuable as a relatively non-toxic means of releasing oligonucleotides from the endosomal

or lysosomal compartments in cells, thus allowing them to reach targets in the cytoplasm and nucleus. Although the difference in IC_{50} between liposomal and free antisense is only a factor of 2, the efficacy and availability of the liposomal oligo is much greater and more specific in inhibiting replication at molecular as well as protein level, which is evident by PCR (Fig. 5) and indirect immunofluorescence assay, respectively (data not shown).

The pandemic threat of HIV infection has prompted intensive research for new antiviral agents. Many compounds are currently being investigated for their efficacy in treating AIDS. Considering the severity of AIDS and the current necessity of long-term chemotherapy, more potent and less toxic antivirals are needed. Conjugates of specific antibody to oligonucleotide-containing liposomes provide enhancement in cell specificity, protection against degradation, and prolonged cellular delivery of oligonucleotides. The altered HIV-1 mRNA specifically induced by targeted antisense DNA liposomes suggests that the mechanism for the inhibition of viral expression is its interaction with the *rev* regulatory gene, resulting in a translation arrest. These liposomal antisense reagents are active at low concentrations, resist nuclease degradation and retain their target sequence specificity. Therapeutic application of this particular targeting system awaits further development in both in vitro and in vivo.

Acknowledgements

The authors wish to thank Drs S. Dhawan, E. Cowan and M. Farshid for their critical review and discussion of this manuscript, and Dr S. Dhawan for providing freshly prepared PBL cells.

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