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Inhibition of Human T-Cell Leukemia Virus Type 1 Replication by Antisense *env* Oligodeoxynucleotide

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Human T-cell leukemia virus type 1 (HTLV-1) infection is associated with adult T-cell leukemia and HTLV-associated myelopathy/tropical spastic paraparesis. Inhibition of HTLV-1 transmission is important to prevent the above HTLV-1-associated diseases. We used the antisense oligodeoxynucleotides (oligos) complementary to the first splice junction, rex responsive site, *gag, env, tax, rex,* and *p21* and evaluated the effects on the syncytium formation between HTLV-1 producing human T-cell line, C91/PL cells, and HTLV-1-uninfected human glioma cell line, U251-MG cells. The syncytium formation was significantly inhibited the virion production assayed by antisense oligos to *env, tax, gag, p21,* and *rex,* with antisense oligo to *env* being the most inhibitory. Antisense oligos to *env* and *tax* also inhibited reverse transcriptase activity. Antisense oligo to *env* may have a potential as a preventive measure of HTLV-1 replication and transmission *in vivo.*

Chronic infection of human T-cell leukemia virus type 1 (HTLV-1) (1,2) is known to be closely associated with adult T-cell leukemia (ATL) (3), HTLV-1 associated myelopathy/ tropical spastic paraparesis(HAM/ TSP)(4,5), and other HTLV-1-associated diseases (6,7). Prevention of HTLV-1 transmission could be achieved at various stages including entry, reverse transcription, integration, transcription, translation, and particle formation.

Previous studies have shown that antibodies against HTLV-1 prevent transmission using experimental animals (8). Recently, antisense oligodeoxynucleotides-(oligos) targeted against various viruses have been successfully designed and show promising results (9-12).

Although HTLV-1 transmission *in vitro* could occur using cell-free HTLV-1 in certain experimental conditions (13,14), introduction of HTLV-1-infected cells are required for HTLV-1 transmission *in vitro* and *in vivo* in most cases (15-20). Thus, cellular contact between HTLV-1-infected cells and HTLV-1-uninfected cells is usually required for efficient HTLV-1 transmission.

Here we used antisense oligos against HTLV-1 sequences to test their inhibitory effects on viral transmission using the HTLV-1-induced syncytium formation assay. We found that antisense oligo to *env* inhibited syncytium formation and viral replication. The mechanism(s) of inhibition of syncytium formation by antisense oligo to *env* is discussed.

MATERIALS AND METHODS

Cells. HTLV-1-producing human T-cell lines, C91/PL cells(19) and MT-2 cells (16), were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum(FBS). HTLV-1-uninfected human glioma cell line, U251-MG cells (21), was maintained in Eagle's minimal essential medium(E-MEM) supplemented with 10% heat-inactivated FBS.

Antisense and control oligos. To study the inhibition of HTLV-1 transmission, phosphorothioate forms of 7 antisense oligos and a control oligo were synthesized (Fig. 1). Five antisense oligos were complementary in sequence to the region of the translation start site of the *tax* (5′AAGTGGGCCATGGTGTTGGA3′), *rex* (5′GGTCTTGGGCATGCAGCTC3′), *gag* (5′GATTTGGCCCATTGCCTAGG3′), *env* (5′AACTTACCCATGGTGTTGGA3′), and *p21* (5′GATAACGCGTCCATCGATGG3′) genes. Two other antisense oligos correspond to the first splice junction (FSJ) (5′GACCAGGAAGCTAGACGGCG3′), and the rex responsive site (RRS) (5′CCCGGTCTCGACCTGAG3′), respectively. In addition, a random oligo was synthesized as a negative

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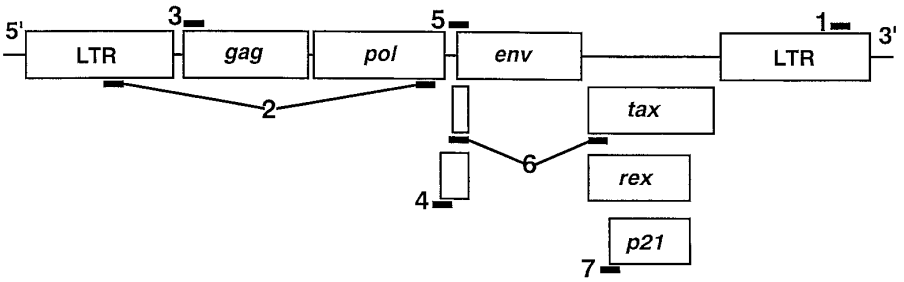

Antisense	1	rex responsive site	: CCCGGTCTCGACCTGAG
	2	1st splice junction	: GACCAGGAAGCTAGACGGCG
	3	*gag*	: GATTTGGCCCATTGCCTAGG
	4	*rex*	: GGTCTTGGGCATGCAGCTC
	5	*env*	: AACTTACCCATGGTGTTGGA
	6	*tax*	: AAGTGGGCCATGGTGTTGGA
	7	*p21*	: GATAACGCGTCCATCGATGG

FIG. 1. Antisense oligos targeted against various regions of the HTLV-1 provirus. The sequences of oligos corresponding to the respective proviral regions are shown by bars with numbers. Nucleotide sequences of oligos are in the 5′ to 3′ direction.

control. All oligos were purified by high performance liquid chromatography.

Assay for cell viability. To examine the toxicity against cell lines, oligos were added at various concentrations. After culturing for 48 h, viable cells were counted by trypan blue exclusion.

Syncytium formation assay. Syncytium formation assay was performed according to Hoshino et al. (22) and Nagy et al. (23). U251-MG cells were seeded in 96 well plates at 2.5 × 104 cells/ml in E-MEM containing 10% FBS. The following day, C91/PL cells were overlaid onto U251-MG cells at 2.5 × 104 cells/ml in RPMI-1640 medium with 10% FBS in the absence or presence of oligo at various concentrations. After culturing for 24 h at 37°C, the cells were fixed with methanol and stained with 5% Giemsa solution. Numbers of syncytia containing more than 10 nuclei were counted. In this assay system, 1000-fold and 100-fold dilution of the plasma from a patient with HAM/TSP caused 52% and 86% inhibition of syncytium formation.

Reverse transcriptase (RT) assay. RT assay was performed according to Poiesz et al. (1). C91/PL cells and MT-2 cells were seeded at 2.5 × 105 cells/ml in RPMI-1640 medium with 10% FBS in the absence or presence of oligo at 10 μM. After culturing for 24 h at 37°C, oligo was again added to a final concentration of 20 μM. After 24 h, culture medium was mixed with 30 μl of 4 M NaCl and 360 μl of 30% polyethylene glycol (Carbowax 6000) and the suspension was placed on ice for 2.5 h. The suspension was centrifuged and the precipitate was resuspended in 20 μl of 50% glycerol, 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.025% Triton X-100, and 5 mM dithiothreitol (DTT), and 10 μl of 0.9% Triton X-100 and 1.5 M KCl. RT assay was performed for 1 h at 37°C with a 10 μl aliquot of the disrupted virus suspension in a final volume of 60 μl containing 40 mM Tris-HCl (pH 7.8), 4 mM DTT, 45 mM KCl, 250 μg/ml bovine serum albumin, 1.8 μg oligo (dT)12-l8 (Pharmacia, Biotech, Tokyo, Japan), 9 μg poly(rA) (Pharmacia, Biotech, Tokyo, Japan), 15 μM [methyl-3H] thymidine 5′-triphosphate (1 Ci/mmol, Du Pont/NEN, USA), and 0.25 mM MnCl2. The assay mixture was spotted onto DE-81 filter paper. The filters were washed with 5% Na2HP04, distilled water and ethanol. The radioactivity of the filters was measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

Like many other retroviruses, HTLV-1 can induce syncytium formation when HTLV-1 infected cells are cocultured with uninfected target cells (22,23). Thus, inhibition of HTLV-1 transmission *in vitro* would be expected to result in the inhibition of syncytium formation. We employed syncytium formation assay to screen the inhibitory activity of various antisense oligos to HTLV-1 (Table 1).

In the syncytium formation assay, seven antisense oligos and a control oligo were added to C91/PL cells and U251-MG cells at 10 μM. No toxicity was observed by counting viable cells (Table 1). Interestingly the antisense oligo to *env* showed the strongest inhibition of syncytium formation. Weaker inhibition was ob-

TABLE 1

Effect of Antisense Oligodeoxynucleotides Phosphorothioates on Syncytium Formation and Cell Growth

Oligos	Number of syncytia (%)[a]	Viable cell number(%)[b]	
		U251-MG	C91/PL
No addition	105.6 ± 4.0	100[f]	100[g]
Random	100.0[e] ± 4.3	98	94
Rex responsive site	111.2 ± 4.8	102	94
First splice junction	100.4 ± 2.5	109	97
gag	46.8 ± 1.6[d]	111	113
rex	66.3 ± 2.5[c]	102	116
env	28.8 ± 0.9[d]	103	101
tax	44.2 ± 4.0[d]	97	95
p21	58.4 ± 1.5[c]	112	116

[a,e] Normalized number of syncytia compared with "Random." Number of syncytia of [e]66.8 was normalized as 100%. C91/PL cells were added with 10 μM oligodeoxynucleotides and were co-cultured with U251-MG cells. Mean ± SE (n = 4).

[b,f,g] Normalized number of viable cells (n = 2). The cell number of [f]2.83 × 10^5 cells/ml and [g]2.78 × 10^5 cells/ml were normalized as 100%. 10 μM oligodeoxynucleotides were added to U251-MG cells and C91/PL cells. After culturing for 48 h, viable cells were counted by trypan blue exclusion.

[c,d] Statistically significant by Student's *t* test. [c]$p < 0.001$, [d]$p < 0.0001$.

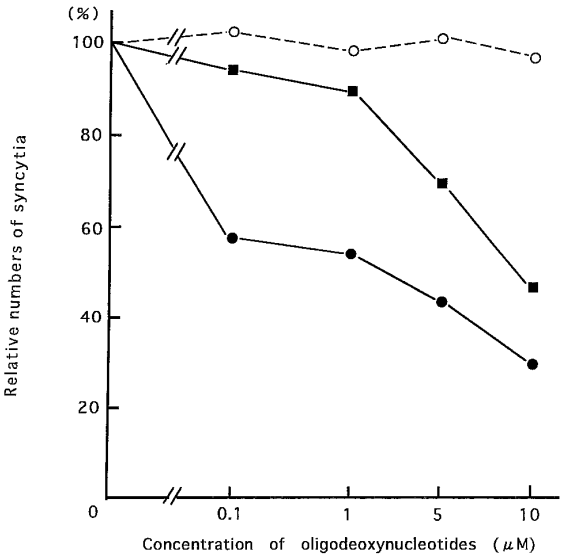

FIG. 2. Dose-response inhibition of syncytium formation by antisense oligos. Oligos at the indicated concentration were added to C91/PL cells and cocultured with U251-MG cells. Random oligo (○), antisense oligo to *env* (●), and antisense oligo to *tax* (■). The numbers of syncytia in the absence of oligo were normalized to 100%. The assays were carried out in duplicate.

served with antisense oligo to *tax*. Antisense oligos to *gag*, *p21* and *rex* also significantly inhibited the syncytium formation, but to a much lesser extent (Table 1). Recently, Miyano-Kurosaki et al. (12), using different cell systems, found several oligos including homooligomer of deoxycytidylate and random oligo inhibited HTLV-1-induced syncytium formation, while antisense oligo to *tax* did not. This inhibition is speculated to be caused by nonspecific binding to cellular components in a nonantisense manner (12). In the present work, antisense oligo to *env* and *tax* inhibited syncytium formation in a dose-dependent manner, while random oligo did not (Fig. 2). Thus the mode of action of these antisense oligos to *env* and *tax* on the syncytium formation in this work seems to be attributable to an antisense manner.

Although the interaction of viral envelope protein and specific cell surface receptors (22,23) is important for syncytium formation, the size or number of syncytia is modified greatly by various factors including cell adhesion molecules (24,25), lipid composition of the target cell membrane (26), activation state of target cells (27-29) and altered glycosylation of membrane antigen in HTLV-1 positive T-cells (25). The present work used a human T-cell line C91/PL and a human glioma cell line U251-MG, while Miyano-Kurosaki et al.(12) used a human T-cell line MT-2 and a rat sarcoma cell line XC, as the HTLV-1 producer cells and target cells, respectively. It is reported that syncytium formation was inhibited by antibodies against ICAM-1 and LFA-1 when a human T-cell line C91/PL and a human T-cell line MOLT4/#8 were used for syncytium formation (30), but it was not inhibited by similar antibodies when a human T-cell line HCT-1 and a rat sarcoma cell line XC were used (31). Similarly the antibody against C33 membrane antigen could inhibit syncytium formation when MOLT-4/#8 was used as the target cells, but it did not inhibit the syncytium formation when another human cell line HOS was used as the target cells (24). Therefore the different combination of cells might be one of the factors to explain the different cellular responses to the oligos, and the apparent discrepancy between the present work and that by Miyano-Kurosaki et al.(12) in the syncytium formation assays.

Although the precise mechanism of action of antisense oligos is not well clarified, inhibition of RNA splicing, inhibition of translation of mRNA, and degradation of RNA by RNase H (32-34) are suggested to be the sites of action. These effects would cause the inhibition of viral proteins and inhibition of virion production, thus causing inhibition of syncytium formation. We then tested the effects of antisense oligo to *env* in virion production, by determining the RT activity of the virions produced in the supernatant of viral producer cell lines. We used two HTLV-1-producing human T-cell lines, C91/PL cells and MT-2 cells. In the RT assay, antisense oligos and a control oligo were added twice to C91/PL cells and MT-2 cells to make a final concentration of 20 μM. The numbers of viable cells in the presence of oligo are comparable to that in the absence of oligo (Table 2),

TABLE 2

Effect of Antisense Oligodeoxynucleotides Phosphorothioates on Reverse Transcriptase Activity and Cell Growth

Oligos	Reverse transcriptase activity (%)[a]		Viable cell number (%)[b]	
	C91/PL	MT-2	C91/PL	MT-2
No addition	114.2 ± 2.5	128.5 ± 7.5	100[g]	100[h]
Random	100.0[e] ± 2.6	100.0[f] ± 3.8	100	94
Rex responsive site	96.5 ± 3.8	92.0 ± 4.8	100	89
First splice junction	101.0 ± 2.6	82.1 ± 4.0	106	94
gag	86.7 ± 4.6	82.9 ± 2.1	106	100
rex	95.9 ± 4.0	94.0 ± 3.6	94	89
env	63.9 ± 2.7[d]	46.7 ± 3.0[d]	100	94
tax	78.2 ± 3.8[c]	74.4 ± 3.3[c]	106	106
p21	90.7 ± 5.2	99.1 ± 5.4	94	100

[a,e,f] Relative activity of reverse transcriptase compared with "Random." Radioactivity, [e]2754 cpm and [f]3630 cpm were normalized as 100%. C91/PL or MT-2 cells were added with 10 μM oligodeoxynucleotides at the start and at the 24th hour of incubation. The reverse transcriptase activity was determined after 48 hours of incubation. Mean ± SE (n = 3).

[b,g,h] Normalized number of viable cells (n = 2). The cell number of [g]8.5×10^5 cells/ml and [h]9.0×10^5 cells/ml were normalized as 100%. After culturing for 48 h, viable cells were counted by trypan blue exclusion.

[c,d] Statistically significant by Student's *t* test. [c]$p < 0.01$, [d]$p < 0.001$.

suggesting that addition of oligo was not toxic to cells under the above conditions. Among seven oligos, antisense oligo to *env* at 20 μM most significantly inhibited RT activity of the supernatant from C91/PL cells and MT-2 cells (Table 2). Antisense oligo to *tax* showed weaker inhibition than that to *env*. Other antisense oligos did not show significant inhibition. We further analyzed the expression of Env by C91/PL cells using monoclonal antibody against gp21 of HTLV-1 Env by flow cytometry. The effects of antisense oligos to *env* and *tax* inhibited the expression of Env antigen by 15% and 14%, respectively, as compared to that of control oligo, but these effects were statistically not significant (data not shown).

Thus the mechanism of inhibition of virion production by antisense oligo to *env* is not clear but might be due to other step(s) than expression of Env. To our knowledge, this is the first indication that antisense oligo to *env* inhibited syncytium formation and HTLV-1 replication. We believe this information is useful for future designing a new strategy for prevention of HTLV-1 transmission *in vivo*.

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