Inhibition of Mouse Hepatitis Virus Multiplication by Phosphorothioate Analogues of Oligodeoxynucleotides Complementary to the Leader RNA

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ABSTRACT. Phosphorothioate oligodeoxynucleotides (PS-oligo) complementary to a leader RNA of mouse hepatitis virus (MHV) were more effective inhibitors of MHV multiplication than natural oligodeoxynucleotides (PO-oligo). Sequence-dependent inhibition of viral multiplication was shown at low concentrations (0.001–0.1 μM) of antisense PS-oligo. Phosphorothioate oligodeoxycytidine, PS-$(dC)_{20}$ and PS-oligo, which has no significant homology to the MHV sequence, showed inhibitory effects on MHV multiplication at concentrations higher than 0.5 μM. These results showed that PS-oligo was more potent than PO-oligo in inhibition of MHV multiplication and that PS-oligo may inhibit MHV multiplication by two different mechanisms, that is, in sequence-dependent and -independent manners. — KEY WORDS: antisense oligonucleotide, MHV, phosphorothioate oligonucleotide.

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Antisense oligodeoxynucleotides within cells targeted toward the RNA transcript of a specific gene can inhibit the expression or promote the degradation of the transcript, resulting in supression of the function coded for by the gene [2, 5]. Antisense oligodeoxynucleotides have also been widely used as tools for inhibiting viral replication [13, 15, 17, 18]. However, the susceptibility to degradation by nucleases of the phosphodiester linkage in natural oligodeoxynucleotides appears to reduce their potency and persistence *in vivo* as antiviral agents [16]. Therefore, considerable efforts have been made to develop chemically stable oligodeoxynucleotides which are nuclease-resistant, but retain their Watson-Crick base pairing specificity. Phosphorothioate oligodeoxynucleotides (PS-oligo), in which a backbone oxygen is replaced with sulfur are more stable against cleavage by nucleases and retain the base pairing specificity [14]. Furthermore, PS-oligo has good solubility since it has the same number of charges as natural phosphodiester oligodeoxynucleotides (PO-oligo). It has been reported that antisense PS-oligo is up to 100 times more potent than PO-oligo of the same sequence in inhibition of gene expression of human immunodeficiency virus (HIV) [1]. However, sequence-independent antiviral activity by PS-oligo has been shown. Phosphorothioate homooligodeoxynucleotides, such as oligodeoxycytidine and PS-oligo containing no homologous sequence with the HIV gene also inhibit replication of HIV at high concentrations [1, 11]. The mechanism of such sequence-independent inhibition of HIV multiplication by PS-oligo remains unclear at present.

Mouse hepatitis virus (MHV), a member of the Coronaviridae, contains a single-stranded, positive-stranded RNA genome *c.a.* 31 kilobases in length [12]. The 5'-ends of the genomic RNA and all mRNA species contain a leader sequence of approximately 70 nucleotides [4, 7]. The conserved sequence in the leader RNA, UC(U/C)AAAC, may play an important role in viral transcription [7, 8]. Previously we reported that an oligonucleotide complementary to a leader RNA containing the conserved sequence of MHV inhibits the viral multiplication [10]. Therefore, we selected the leader sequence including the conserved sequence as a target region for antisense PS-oligo and investigated the effect of PS-oligo on MHV multiplication.

DBT cells [6] were cultivated in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS) at 37°C in a humidified atmosphere containing 5% CO_2. DBT cells were infected with the JHM strain of MHV (JHMV) [10] at a multiplicity of infection (m.o.i.) of 0.1 in the absence or presence of PS-oligo at concentrations from 0.001 to 5 μM for 1 hr at 37°C, in CS-free MEM. After the incubation, PS-oligo and the virus were removed, and then cells were washed twice with CS-free MEM. After the addition of MEM with CS, plaque assays were performed to titrate infectious progeny in the supernatants at each time post infection (p.i.) according to the method of Hirano *et al.* [6]. The infectivity was expressed as the percentage of inhibition = (1 – PFU obtained from cells treated with PS-oligo/PFU obtained from control cells untreated with PS-oligo) × 100 (%).

Phosphorothioate oligodeoxynucleotides were obtained from Sawadii Tech. Co. (Tokyo). AL-oligo (5'AAAGTTTAGATTAGATTAGA3') contained a sequence complementary to the conserved sequence of a leader RNA of JHMV (Fig.1). ML-oligo (5'AAAGTTAATGTAATGTTAGA3') contained a sequence with 70% homology to AL-oligo. Random-oligo (5'ACCCGTAGCTAATGAGATAT3') contained no significant homology with MHV sequences as yet reported. Phosphorothioate oligodeoxycytidine, PS-$(dC)_{20}$ (5'CCCCCCCCCCCCCCCCCCCC3') was also synthesized.

DBT cells were incubated with JHMV (0.1 m.o.i.) in the presence of PS-oligo at concentrations from 0.001 to 5 μM for 1 hr at 37°C. The treatment of the cells with PS-oligo had no apparent detrimental effect on cell viability (data not shown). A typical result of a plaque assay is shown in

5'-AUCUAAUCUAAUCUAAACUUUA-3'
60 70 80

Oligonucleotide	Sequence 5' - 3'
AL-oligo	AAAGTTTAGATTAGATTAGA
ML-oligo	AAAGTTAATGTAATGTTAGA
Random-oligo	ACCCGTAGCTAATGAGATAT
PS-$(dC)_{20}$	CCCCCCCCCCCCCCCCCCCC

Fig. 1. Sequences of the leader RNA containing conserved sequence UC(U/C)AAA and PS-Oligo.

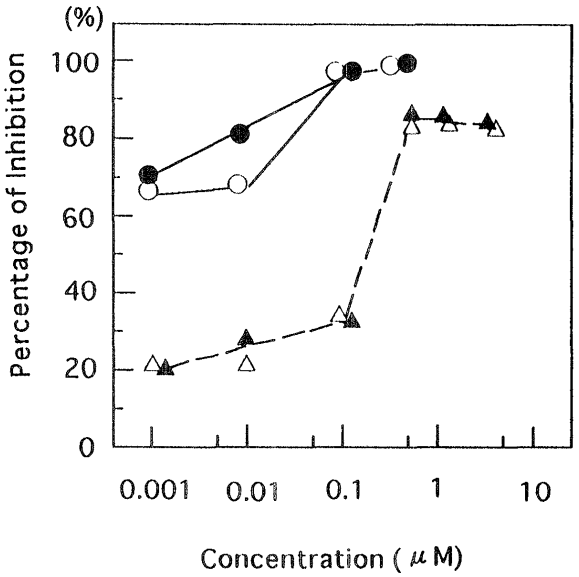

Fig. 2. The effects of PS-oligo on MHV multiplication. DBT cells were infected with JHMV (0.1 m.o.i.) in the presence of AL-oligo (○), ML-oligo (●), Random-oligo (△), and PS-$(dC)_{20}$ (▲) for 1 hr at 37°C. Plaque assays were performed to titrate infectious progeny in the supernatants at 12 hr p.i. Each point represents an average of three separate experiments.

Table 1. Inhibitory effects of PS-oligo on MHV multiplication

Oligonucleotide	Concentration (μM)	hr p.i.	PFU/m*l*	Percentage of Inhibition (%)
None	0	12	7.2×10^5	–
AL-oligo	0.1	12	3.6×10^4	95
ML-oligo	0.1	12	3.1×10^4	96
Random-oligo	0.1	12	4.6×10^5	36
PS-$(dC)_{20}$	0.1	12	4.5×10^5	38
None	0	24	2.9×10^6	–
AL-oligo	0.1	24	1.3×10^6	55
ML-oligo	0.1	24	1.9×10^6	36
Random-oligo	0.1	24	3.5×10^6	-19
PS-$(dC)_{20}$	0.1	24	2.8×10^6	3

Table 1. The yields of infectious virion particles from the cells treated with AL-oligo and ML-oligo at 0.001 μM were reduced significantly compared with the yields from control cells untreated with PS-oligo (Fig 2). At 0.1 and 0.5 μM the viral multiplication was inhibited more than 95%. Since no inhibitory effect on the viral multiplication was observed at 1 μM after treatment with natural PO-oligo complementary to the leader RNA [10], PS-oligo was 1,000 times more potent than unmodified PO-oligo. It has been reported that PS-oligo is more resistant to nuclease digestion in cells and in the whole body [3, 14]. Therefore, PS-oligo might more effectively inhibit viral multiplication in infected cells than PO-oligo did. Although ML-oligo contained a sequence only 70% homologous to AL-oligo, no significant difference was observed in inhibitory effects on MHV multiplication between AL-oligo and ML-oligo. The reason why there was no significant difference between AL-oligo and ML-oligo in spite of the difference of homology remains unclear. It is well known that the sequence of the 5' end is important in hybrid formation between the oligonucleotide and the template. Since AL-oligo and ML-oligo have the same sequences at both 5' and 3' ends, the efficiency of hybrid formation between AL-oligo and the leader RNA may be similar to that between ML-oligo and the leader RNA.

Random-oligo which contained no sequence with significant homology to MHV genes and oligodeoxycytidine, PS-$(dC)_{20}$, showed inhibitory effects on viral multiplication (Fig. 2). The percentages of inhibition by random-oligo and PS-$(dC)_{20}$ were significantly lower than those by AL-oligo and ML-oligo at low concentrations. These results suggested that PS-oligo may inhibit the viral multiplication in a sequence-dependent manner at a low concentration. At concentrations higher than 0.5 μM, viral multiplication was inhibited more than 80%. Thus, sequence-independent inhibition was observed at a high concentration of PS-oligo. The sequence-independent inhibition of HIV multiplication has been reported at a high concentration [1, 11]. The present results showed that such a sequence-independent inhibitory effect on viral multiplication by PS-oligo was not specific to HIV. A study of the mechanism of this sequence-independent inhibition is now in progress.

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