Inhibition of Viral Multiplication in Cells Chronically Infected with Mouse Hepatitis Virus by Antisense RNA against the Polymerase Gene

Akihiko MAEDA, Masanobu HAYASHI, Tetsuya MIZUTANI, Kozue ISHIDA, Tomomasa WATANABE, and Shigeo NAMIOKA
Department of Laboratory Animal Science, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan
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ABSTRACT. Recently, we showed that the antisense RNA containing a hammerhead ribozyme sequence against the polymerase gene of mouse hepatitis virus (MHV) inhibited viral multiplication in acute infection [10]. In the present study, we examined the inhibitory effects of an antisense RNA on viral multiplication in chronic MHV infection. In cell line LR-2, in which the 926-nucleotide (nt) antisense RNA containing a ribozyme sequence against the polymerase gene was expressed constitutively at a high level, chronic MHV infection was established through the maintenance of infection over 100 days postinfection (d.p.i.). After 200 d.p.i., no infectious progeny virus was observed in the culture medium of chronically MHV infected LR-2 cells. Our present results showed that the antisense RNA could also inhibit viral multiplication in chronic MHV infection.—KEY WORDS: antisense RNA, chronic infection, mouse hepatitis virus.

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Recently, antisense nucleic acids including ribozymes that catalyze the cleavage of their target RNAs and many other reactions in a sequence-specific manner [2, 16], have been used as regulatory agents of gene expression [3, 15], viral replication [19] and cancer development [17]. Although antisense agents inhibit viral multiplication effectively in acute infection, escaped viruses may be produced in chronically infected cells. For example, in cells chronically infected with human immunodeficiency virus (HIV), the escaped viruses are produced through long-term treatment with antisense oligonucleotides against HIV [9, 13]. The production of escaped viral particles may be due to the mutation of viral genes, since HIV shows high mutation rates [7].

Mouse hepatitis virus (MHV) is a member of the *Coronaviridae*, which causes a variety of diseases including hepatitis and encephalomyelitis in laboratory mice [4, 21]. MHV is an enveloped virus with an approximately 31 kilobase (kb) positive-stranded, genomic RNA [8] and shows a high mutation rate [1]. *In vivo* and *in vitro*, chronic MHV infection has been established [6].

We have reported that multiplication of MHV is significantly inhibited in acute infection by the antisense RNA complementary to the nucleocapsid (N) protein gene of MHV [14] and the antisense RNA containing a hammerhead ribozyme sequence targeted against the polymerase gene of MHV [10]. However, whether the antisense RNA inhibits the viral multiplication remains unknown in cells chronically infected with MHV. In a previous report [10], we established LR-2 cells in which the 926-nucleotides (nt) antisense RNA complementary to +224 to +1150 sequence of the polymerase gene of MHV-JHM, which contains a 22-nt hammerhead ribozyme sequence, was expressed constitutively at a high level, and viral multiplication was significantly inhibited in acute MHV infection. To examine the inhibitory effects of the antisense RNA containing a hammerhead ribozyme against the polymerase gene of MHV on viral multiplication in chronic MHV infection, we established LR-2 cells chronically infected with MHV and examined viral multiplication.

Mouse astrocytoma-derived DBT cells [5] and LR-2 cells [10] were grown in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum (CS) and 2 mM glutamine. The cells were kept in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C. The JHM strain of MHV used in this study was propagated in DBT cells [11].

Cells chronically infected with MHV were established essentially according to the methods of Hirano *et al.* [6]. Briefly DBT cells and LR-2 cells were infected with MHV-JHM at a multiplicity of infection (m.o.i.) of 0.1. At 12 hr postinfection (hr p.i.), fusion-escaped cells were harvested, washed twice with PBS(−), filtrated with gauze, replated on new 6-cm culture plates, and then cultured in MEM with 5% CS. Confluently grown cells were harvested and replated onto other plates. The infected cells that were passaged in this way 16 times or more were regarded as chronically infected cells. To titrate infectious progeny viruses in the culture medium at each postinfection time, plaque assays were performed according to the method of Hirano *et al.* [6].

Cytoplasmic RNAs were extracted from infected DBT cells and LR-2 cells according to the method of Silver *et al.* [20]. After ten-microgram RNA samples were electrophoresed in 1.0% agarose gel containing 5.5% formamide, the RNA molecules were transferred onto nitrocellulose membranes by the method of Maniatis *et al.* [12]. cDNA of mRNA7 of MHV-JHM and cDNA corresponding to nucleotides +224 to +1150 sequence of the genomic RNA of MHV-JHM containing the ribozyme core sequence [10] were radiolabeled with [α-^{32}P]dCTP by nick-translation [18] for use as probes. Prehybridization, hybridization and washing were carried out as described previously [10].

Although antisense nucleic acids including ribozymes are used as inhibitory agents of viral multiplication, it has been shown that escaped viruses are produced through long-term treatment with antisense nucleic acids [9, 13]. The production of escaped viruses might be caused by a mutation of viral genes through the long-term infection. To examine the effects of antisense nucleic acids on viral multiplication in chronic MHV infection, we have established several cell lines expressing antisense RNAs con-

taining a hammerhead ribozyme sequence targeted against the RNA polymerase gene of MHV. In LR-2 cells expressing a 926-nt antisense sequence with a 22-nt ribozyme sequence, MHV multiplication was effectively inhibited in acute infection [10]. Therefore, we used LR-2 cells for the examination of the effect of the antisense RNA containing a ribozyme sequence on MHV multiplication in chronic infection in this study.

When infectious progeny viruses in the culture medium of chronically infected DBT cells and LR-2 cells were titrated at 95 to 106 days postinfection (d.p.i., from 16 to 17 passages) by plaque assay, the yields of progeny viruses produced from LR-2 cells were significantly reduced as compared to those from DBT cells (Fig. 1). No viral particle was observed at 200 and 250 d.p.i. in LR-2 cells (Table 1). These results showed that the antisense RNA

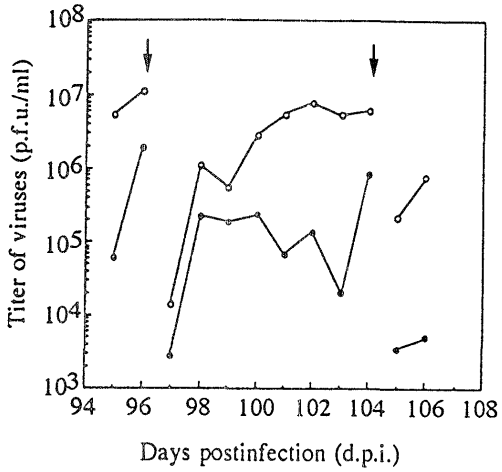

Fig. 1. Yields of infectious progeny viruses in chronically infected DBT and LR-2 cells. Infectious progeny viruses of the supernatant of chronically infected DBT (○) and LR-2 cells (●) from 95 to 106 d.p.i. were titrated by plaque assay. Confluently grown cells were harvested and replated on other plates at the times indicated by arrows.

Table 1. Yields of viral particles in infected DBT cells and LR-2 cells

Infection	Cells	Yields of viruses (p.f.u./ml)
9 hr p.i.	DBT	$3.39\pm0.34\times10^4$
	LR-2	$9.27\pm0.60\times10^3$
104 d.p.i.	DBT	$5.87\pm0.26\times10^6$
	LR-2	$0.83\pm0.23\times10^6$
200 d.p.i.	DBT	$3.92\pm1.49\times10^6$
	LR-2	ND
250 d.p.i.	DBT	$1.81\pm0.24\times10^6$
	LR-2	ND

Each value represents the average of four separate experiments ± standard deviation (S.D.). ND represents not detected.

containing a ribozyme sequence effectively inhibited MHV multiplication in chronic infection. Northern blot analysis showed antisense RNAs containing a ribozyme sequence in LR-2 cells at 104 and 200 d.p.i. (lane 4 in Figs. 2b and c). This result showed that this antisense RNA containing a ribozyme sequence was stably expressed in chronically infected LR-2 cells. Since genomic RNA and all subgenomic mRNAs contain a sequence complementary to mRNA7, all mRNA species were hybridized with cDNA of mRNA7 (lane 1 in Fig. 2). The amounts of viral RNAs in LR-2 cells were also significantly reduced at 104 and 200 d.p.i. as well as at 9 hr p.i. as compared to those in DBT cells (Fig. 2).

The production of escaped viruses might be due to a mutation of the viral gene targeted against antisense nucleic acids during long-term treatment. The escape may occur more easily against short (15-nt) antisense nucleic acids than against longer ones (20-nt) [9]. In this experiment, no virus production was observed at 200 d.p.i. in LR-2 cells. Since the antisense RNA used in this study contained a long sequence (926-nt), it may be hard for

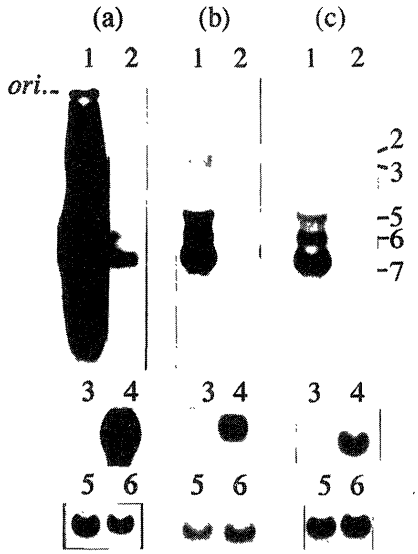

Fig. 2. Detection of the MHV RNA in infected DBT and LR-2 cells by Northern blot analysis. Cytoplasmic RNAs were extracted from DBT cells (lanes 1, 3 and 5) and LR-2 cells (lanes 2, 4 and 6) infected with MHV-JHM at 9 hr p.i. (a), 104 d.p.i. (b) and 200 d.p.i. (c). Lanes 1 and 2 were hybridized with cDNA of mRNA7 of the JHM strain of MHV as a probe. Lanes 3 and 4 were hybridized with cDNA corresponding to nucleotides 224 to 1150 of genomic RNA of MHV containing the ribozyme core sequences as a probe. Lanes 5 and 6 were hybridized with cDNA of β-actin as a probe. The numbers of MHV-mRNAs are indicated to the right of lane 2.

MHV to escape from the antisense effect in spite of the long-term infection.

Although we showed that the ribozyme used in this study cleaved target MHV-RNA at the expected site in a cell-free reaction [10], we could not observe the cleavage products in the transfected cells expressing ribozymes in chronic MHV infection. The target site of the antisense sequence was much longer than that of the ribozyme catalytic site. Therefore, the inhibitory effect seems to be mainly due to the antisense sequence rather than the catalytic core sequence of the ribozyme in chronic infection. In LR-2 cells, viral multiplication is effectively inhibited in acute MHV infection (Table 1 and reference 10). The inhibition of viral multiplication in the early stage of infection might affect the production of viral particles in the chronic stage of infection.

Our present study showed that long antisense nucleic acids might be good antiviral therapeutic agents in chronic infection as well as in acute infection.

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