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Inhibitory Effects of an Antisense Oligonucleotide in an Experimentally Infected Mouse Model of Influenza A Virus

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The antiviral effects of a 20-mer antisense phosphorothioate oligonucleotide, PB2-as, on influenza A virus infection in mice were examined and compared to those of PB2-as encapsulated with several cationic liposomes. Intravenous injection of PB2-as, as a complex with DMRIE-C, a cationic liposome, was most effective for prolonging the mean survival time in days (MSDs) and increasing the survival rates of mice infected with the influenza A virus. In addition, the liposomal PB2-as significantly inhibited viral growth in lung tissues. These results suggest that PB2-as encapsulated with DMRIE-C may be active against the influenza A virus infection through the inhibition of virus replication in the mouse lung.

Key Words: **antisense oligonucleotides; animal model; transfection reagent; influenza A virus; viral RNA polymerase gene; gene therapy.**

Synthetic antisense oligonucleotides have been widely used as tools for the specific inhibition of gene expression. They are considered to be a potential new generation of drugs (1). Antisense oligonucleotides can inhibit various viral pathogens and regulate specific gene expression by inhibiting transcription or translation through their complementary interactions with targeted genetic segments (2). However, there are some problems with the use of oligonucleotides, in that cells are not very permeable to oligonucleotides and oligonucleotides are not stable for *in vivo* applications (3). Factors such as oligonucleotide stability, cellular uptake, subcellular availability, and other pharmacokinetic parameters lead to relatively poor delivery to the targeted molecular sites, and make the treatment cost prohibitive. Thus, transport and intracellular delivery are important considerations when developing an effective oligonucleotide-based therapy. Liposome delivery is one technique that addresses these concerns. Attempts to use liposomes in the delivery of antisense oligonucleotides have been reported (4–6). Cationic lipids are being used *in vitro* and *in vivo* to deliver oligonucleotides for therapeutic or research purposes, and are the vehicles of choice for some gene therapy protocols (7, 8). We have made considerable efforts to design phosphorothioates and to develop delivery techniques, such as the use of the cationic liposome, Tfx-10, for *in vitro* and *in vivo* studies (9–12). PB2-as is an antisense phosphorothioate oligonucleotide containing the AUG initiation codon sequences as targets of PB2 of the influenza A virus RNA polymerases. PB2-as has a highly specific suppressive activity against influenza A virus growth than the other antisense phosphorothioate oligonucleotide targeted to RNA polymerases (PB1 and PA) and nucleoprotein (NP). In this report, we analyzed the anti-viral effects of PB2-as, encapsulated with several types of cationic liposomes, in influenza A virus-infected mice.

MATERIALS AND METHODS

Oligonucleotides. Phosphorothioate oligonucleotides were synthesized by Yuki Gosei Co. Ltd. (Tokyo, Japan). The DNA sequences of these oligonucleotides are as follows. PB2-as (antisense): 5′>TTCTTTCCATATTGAATATA<3′, and PB2-ran (random): 5′>CTTCTTATATACGATTATAT<3′. The oligonucleotide derivatives were purified by reverse phase HPLC on a TSK gel DEAE-5PW column, and were eluted by a 1 M $NaClO_4$ gradient in 20 mM Tris–HCl (pH 8.0).

The following cationic liposomes, which are commercially available trasfection-reagents, were used: FuGENE-6 (blended lipid with other compounds in 80% ethanol, Boehringer Mannheim, GmbH, Germany), DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate, Boehringer Mannheim}, Tfx-10 {*N*,*N*,*N*′,*N*′-tetramethyl-*N*,*N*′-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide and L-dioleoylphosphatidylethanolamine (DOPE) in a molar ratio of 1: 9 (Promega, Madison, WI). DMRIE-C (1,2-dimyristyloxy-propyl-3-dimethylhydroxy ethylammonium bromide and cholesterol in a molar ratio of 1:1 (Gibco, Gaithersburg, MD).

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TABLE 1

Effect of Oligonucleotide-Liposome Complexes on Mean Survival in Days and Survival Rate in Influenza A Virus-Infected Mice

Compounds	Dose (mg/kg)	Free (PBS)	FuGENE-6 (1 mg/kg)	DOTAP (1 mg/kg)	Tfx-10		DMRIE-C		
					1 mg/kg	5 mg/kg	1 mg/kg	5 mg/kg	25 mg/kg
Control	—	4.0 ± 0.5 [0]	4.2 ± 0.5 [0]	3.7 ± 0.4 [0]	4.1 ± 0.5 [0]	3.9 ± 0.4 [0]	4.2 ± 0.8 [0]	4.3 ± 0.5 [0]	4.3 ± 0.5 [0]
PB2-as	20	4.5 ± 0.7 [0]	4.7 ± 0.5* [0]	5.3 ± 0.5* [0]	8.1 ± 0.7* [25]*	8.5 ± 0.6* [27]*	9.2 ± 0.8* [33]*	11.7 ± 0.7** [43]*	12.7 ± 0.5** [57]**
	40	5.5 ± 0.5* [0]	5.7 ± 0.6* [0]	6.6 ± 0.4* [0]	9.5 ± 0.6* [25]*	11.1 ± 0.7* [45]*	11.3 ± 0.8* [33]*	12.5 ± 1.2** [55]**	13.1 ± 1.1** [74]**
PB2-ran	40	3.9 ± 0.4 [0]	4.8 ± 0.7 [0]	4.3 ± 0.5 [0]	3.9 ± 0.4 [0]	4.1 ± 0.5 [0]	4.2 ± 0.4 [0]	4.0 ± 0.6 [0]	4.7 ± 0.5 [0]
Ribavirin	40	13.5 ± 1.3** [85]**							

Note. Data represent the means ± standard deviations of survival in days. Brackets indicate the percent of survival on the 14th day after the virus infection. $*P < 0.05$, $**P < 0.01$, compared to the control group of mice with vehicle treatment (mean survival in days, *t*-test; survival rate, χ^2 analysis). $N = 6–10$ per group.

Two or four mgs of the oligonucleotides were mixed with 1 mg of liposomes in 1 ml PBS at room temperature for 15 min, as recommended by the supplier. Ribavirin [1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide] was provided by Yamasa-Shoyu Co. Ltd. (Chiba, Japan).

Mice. Female specific-pathogen-free BALB/c mice, weighing 17 to 19 g (6-weeks-old) each, were obtained from Japan SLC Inc. (Shizuoka, Japan). All mice were kept at constant temperature (25 ± 1°C) and humidity (555%) under conditions of a 12–12 h light-dark cycle throughout the experimental periods. This study was carried out under the control of the Animal Research Committee, in accordance with the Guidelines on Animal Experiments at Fukushima Medical College and the Japanese Government Animal Protection and Management Law (No. 105).

Virus. Influenza A/PR/8/34 (H_1N_1) and B/Norway/1/80 viruses were initially obtained from the American Type Culture Collection. The viruses were passed 12 times through mice and were used as a mouse lung homogenate to enhance the *in vivo* virulence. The murine infectivity of the virus was determined with mice weighing 17 to 19 g exposed intranasally (i.n.) to various dilutions of the virus.

In vivo administration of oligonucleotides. Mice were anesthetized by ip administration of pentobarbital-Na at 50 mg/kg (Abbott Laboratories) and were inoculated i.n. with 40 l of a virus suspension containing 100 LD_{50}s. The mice were pretreated iv with a 0.2 ml vol of liposome–oligonucleotide complexes at 24 and 12 h before the i.n. challenge of the mouse-adapted influenza A virus (days −1), and were posttreated iv twice (9 a.m. and 6 p.m.) daily on days 0 to 4. The complexes of PB2-as or PB2-ran (negative control) were used at doses of either 20 or 40 mg/kg, and those of the cationic liposomes were either 1, 5, or 25 mg/kg. Ribavirin (positive control) at 40 mg/kg was also injected iv into a group of infected mice twice daily on days −1 to 4. The observation period was over 14 days postinfection, and we assessed the efficacy in terms of the reductions in mortality.

Lung virus assays. To determine the virus titer in the lung, the mice were sacrificed on day 4. Various 10-fold dilutions of the lung homogenate were quantified at six times for infectious virus by the plaque-forming assay with Madin–Darby canine kidney (MDCK) cells. The fifty percent tissue culture infective dose ($TCID_{50}$) was determined from the linear portion of a log dose versus percentage infection plot by best fit regression analysis. Percent survival was determined by a χ^2 analysis. MSDs and virus titers were analyzed by the Student's *t* test.

RESULTS AND DISCUSSION

Influenza A viruses can cause acute respiratory disease with significant morbidity and mortality. Vaccines have been of limited success thus far, and their effectiveness varies with changes in the major surface antigens of the virus. Furthermore, since therapeutic agents such as amantadine and ribavirin have toxicities, there is a need for improved therapies for this important disease. To overcome these problems, we developed a new antiviral strategy that uses antisense S-ODNs directed against the growth regulatory genes of influenza A viruses in animal models. Antisense oligonucleotides, which are usually targeted to messenger or viral RNAs, decrease the target mRNA level and specifically inhibit protein synthesis. These oligonucleotides specifically bind to complementary sequences of either genomic DNA or genomic RNA through hydrogen bonding of base pairs. However, a problem in the use of antisense S-ODNs is their inefficient cellular uptake. They are mainly found in endosomes and lysosomes. We investigated the protective effects of PB2-as, encapsulated with several types of cationic liposomes, in influenza A virus-infected mice.

As shown in Table 1, when mice were infected with 100 LD_{50}s of influenza A virus, the control mice treated intravenously (iv) with all liposomes showed MSDs of 3.7 to 4.3 days and 0% survival. This concentration of liposome showed no *in vivo* toxicity. PB2-as at 40 mg/kg significantly prolonged the MSDs of the infected mice. The protective effect of PB2-as was more enhanced when the infected mice were treated iv with the complex of liposomes. Especially, DMRIE-C showed the highest efficacy among these liposomes. Mice treated with the complexes of PB2-as (20, 40 mg/kg) and DMRIE-C (25 mg/kg) showed MSDs of 12.7 and

13.1 ($P < 0.01$) days, respectively, and 57 and 74% ($P < 0.01$) survival rates. PB2-as/DMRIE-C complexes showed increased protective activity with an increase in the liposome, DMRIE-C. On the other hands, the Tfx-10 showed slightly weaker protective activity than DMRIE-C. However, FuGENE-6 and DOTAP showed no protective activity. Furthermore, the liposomally encapsulated control oligonucleotide, PB2-ran at 40 mg/kg had no effect on either the MSDs or the survival. In our previous study, the complexes of PB2-as and Tfx-10 (5 mg/kg) showed slightly weaker protective activities than ribavirin. In addition, overdosage of Tfx-10 at 5 mg/kg had toxicity, as manifested by reduced body weights and MSDs in the influenza A virus-infected mice (13). In contrast, DMRIE-C had no toxicity at a dose of 25 mg/kg and showed more efficacy than Tfx-10. The protective effect of PB2-as in the presence of DMRIE-C was close to that of ribavirin (Table 1). On the other hand, the protective effect of the liposomally encapsulated PB2-as was not observed in mice infected with the influenza B virus, B/Norway/1/80 (12). The liposomally encapsulated PB2-as also failed to inhibit influenza B virus proliferation in the lung tissues (12). The PB2 mRNA sequence (20-mer) around the AUG initiation codons of the influenza A virus that are the target sites of PB2-as are UAUAUUCAAUAUGGAAAGAA, and that the influenza B virus is CGUUUUCAAGAUGACAUUGG. The nucleotide sequence identity between the PB2 mRNAs of the influenza A and B viruses is very low (14, 15). The present results suggest that the liposomally encapsulated antisense S-ODN conferred sequence specific inhibition. It has been reported that DNA delivery was enhanced by cholesterol in *in vivo* applications, and this enhancement may be due to its ability to stabilize the liposome bilayer (16–19). These results suggest that the cholesterol within DMRIE-C may contribute significantly to its higher activity as compared to the other liposomes.

To characterize the inhibitory effects of PB2-as, we determined the influenza A virus titers in the lungs of mice treated with PB2-as at 40 mg/kg in the presence of DMRIE-C (5 mg/kg) twice daily for 4 days postinfection. As shown in Table 2, the virus titers in the lungs of the liposomal PB2-as-treated group of mice were 10-fold or more lower than those in the lungs of the control mice treated with the vehicle of DMRIE-C. The degree of reduction induced by PB2-as was the same as that induced by ribavirin at 40 mg/kg. In the negative control group, treatment with PB2-ran at 40 mg/kg had no influence on the virus titers in the lungs. This result suggests that liposomally encapsulated PB2-as has a specific effect against the influenza A virus growth in lung tissues. Studies of the iv administration of liposome-DNA complexes and observations of DNA delivery in many tissues have revealed that the lung invariably shows the highest expression levels. This may be due to a first-passage effect, because the lung is the first capillary bed encountered by the liposome-DNA complex after iv administration (20–22). The present study indicates that the iv administration of PB2-as endocapsulated with DMRIE-C may be useful as a potential therapeutic treatment for influenza A virus disease.

TABLE 2

Efficacy of DMRIE-C Encapsulated Oligonucleotides against Influenza A Virus Titers in Mouse Lung

Compounds	Dose (mg/kg)	Virus titers ($\log_{10}$ $TCID_{50}$s/g lung tissues)
Control	—	8.55 ± 0.40
PB2-as	40	7.05 ± 0.30***
PB2-ran	40	8.72 ± 0.43
Ribavirin	40	7.11 ± 0.44***

Note. $TCID_{50}$ values determined at six time measurements with the lung homogenate obtained from a mouse. Data represent the means ± standard deviations for six mice. Peak virus titers were detected on the 4th day after iv treatment. ***$P < 0.001$, compared to the control group of mice with vehicle treatment (*t*-test).

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