Advances in Microbiology, 2016, 6, 462-470
Published Online May 2016 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2016.66045

Japanese Encephalitis Virus Replication and Inhibitory Effect of shRNA in Mice

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Received 2 May 2016; accepted 17 May 2016; published 20 May 2016

Abstract

Japanese Encephalitis Virus (JEV) is responsible for over 30,000 annual cases of encephalitis worldwide, causing 30% mortality. JEV is thus a continuing threat to public health, so development of new antiviral drugs against JEV is desirable. Here, we examined JEV replication in mouse and used a short hairpin RNA JRi as the antiviral agent. The features of virus replication in neuron and survival rates of mice infected with JEV were different between virus strains. The mice infected with the virulent JEV strain (JaGAr01) were injected with pJRi (100 μg/mouse) which produced shRNAJRi. The survival rates of mice treated at 3 days before, the same day and 3 days after JEV infection were 22%, 78% and 44%, respectively. In addition, we demonstrated that the injection of pJRi induced interferon (IFN) production in cells and mice. These results suggest that the replication of JEV can be efficiently inhibited by RNAi and innate immunity including IFN. These data mean that pJRi has the inhibitory activity against JEV infection *in vivo,* and could be used as an antiviral drug to treat JEV infection.

Keywords

Japanese Encephalitis Virus, Mice, shRNA, Antiviral

1. Introduction

Japanese Encephalitis Virus (JEV) is a member of the flavivirus and small (40 - 60 nm) enveloped virus family

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How to cite this paper: Murakami, M., Tasaki, T., Nukuzuma, S., Minato, H., Nojima, T., Ishigaki, Y. and Takegami, T. (2016) Japanese Encephalitis Virus Replication and Inhibitory Effect of shRNA in Mice. *Advances in Microbiology*, **6**, 462-470.
http://dx.doi.org/10.4236/aim.2016.66045

with a single-stranded positive-sense RNA genome of approximately 11 kb length [1]-[4]. The genomic RNA, which contains a cap structure at the 5'-end and a specific tertiary structure at the 3'-end, encodes a single polyprote which is processed into three structural and seven nonstructural proteins. The viral structural proteins are encoded by the 5'-third of the Open Reading Frame (ORF), and consist of the capsid (C), membrane (M; formed by proteolytic cleavage of its precursor protein prM) and envelope (E) proteins. The nonstructural proteins (NS1 to NS5) are encoded by the remaining 3' region of the ORF. The ORF is flanked by 5'- and 3'-untranslated regions (UTR), which were approximately 95 and 582 nt long, respectively [5].

The mosquito-borne flavivirus, such as JEV, is one of the most important examples of emerging and resurging pathogens, as shown in Zika virus outbreak [6]-[8]. JEV is responsible for over 30,000 annual cases of encephalitis worldwide, causing 30% mortality and permanent neurological disabilities in 50% of survivors [4]. The virus has a normal transmission cycle between mosquitoes and pigs and/or water birds, but also has a zoonotic transmission cycle with swine serving as the amplifier hosts from which the infected mosquitoes transmit the virus to humans. In Japan, it is important to monitor the distribution of JEV, although there have been less than 10 JE cases each year since 1992 [4]. In order to avoid future JE outbreaks, it is necessary to carefully monitor changes in virulence. In the last two decades, we have isolated JEV, Ishikawa strains (genotype 1) from mosquitoes collected in Ishikawa prefecture area in Japan [4]. Interestingly, newly isolated JEV Ishikawa strain belongs to genotype 1 based on the sequencing analysis, whereas all of the JEV strains isolated in Japan before 1990 belonged to genotype 3 [4] [9]. JEV vaccines, however, are effective against all kinds of JEVs including genotype 1 and 3. Although an effective vaccine is available for JEV, multiple doses are required to confer protective immunity, and some pathogenic side effects have also been reported [8]. JEV is thus a continuing threat to public health. Thus, development of new remedies and antiviral drugs against JEV is highly desirable.

RNA interference (RNAi) is an evolutionarily conserved mechanism in which double stranded RNA (dsRNA) induces sequence-specificgene silencing [10] [11]. In mammalian cells, RNAi can be induced via transfection of synthetic siRNAs or DNA plasmids expressing short hairpin RNAs (shRNAs). We have already shown antiviral activity against JEV of 3 different types of RNAi (JCR, JN3R and JRi) in HepG2 and Vero cells [12]. The antiviral activity of JRi *in vitro* was relatively higher than those of the JCR and JN3R. In this paper, we report that the difference in virulence between JEV strains and the antiviral activity of the JRishRNA in mice, and show its effect on interferon (IFN) production *in vivo*.

2. Materials and Methods

2.1 Plasmids

The pJRi plasmid expressing the short hairpin RNA (shRNA) JRi was used for cell transfection and injection into mice. JRi is corresponded to the prM region of the JEV genome. The pJRi plasmid was constructed as previously described [12]. Synthetic oligonucleotides corresponding to JEV genomic RNA sequences were inserted into the pSilencer vector (Ambion, USA) to create pJRi. We used pSilencer and pcDNA-FLAG as control plasmids.

2.2. Viruses, Mice and Cell Lines

The JEVs, JaGAr-01 and Ishikawa strains were used in this study. Pathogen-free female ICR mice, 4 weeks old (average body weight of 21.0 g), were purchased from Sankyo Lab, (Tokyo, Japan). The mice were housed in animal quarters with controlled temperature (24˚C), humidity (50% - 60%), and lighting (12 hrcycle). Vero, HepG2 and IMR32 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 or 10% FBS, 50 units/ml of penicillin and 50 μg/ml streptomycin. Culture cells were incubated at 37˚C with 5% CO_2.

2.3. Viral Titration

The virus yields in the cultured fluids were estimated using the plaque method with baby hamster kidney (BHK) cells, as described previously [13]. A BHK cell monolayer was infected with JEV and then cultured in the presence of 0.6% methylcellulose and 1% FCS in E-MEM. After 3 - 4 days post infection (dpi), the infected cells were fixed using methanol and stained using 1% crystal violet. The viral titer was expressed as plaque-forming units (PFU).

2.4. Viral Challenge Assay Using ICR Mice

A hydrodynamic-transfection method [14] was used to inject 2.0 ml of saline solution containing one of the plasmids (pJRi, pSilencer or pcDNA-FLAG) into the tail veins of the mice. The plasmids were injected into the mice on 3 days before (−3 d), on the same day (0 d) or 3 days after (+3 d) they were infected with JEV (5000 PFU/0.2 ml saline) by the intraperitoneal (i.p.) route. We used 9 mice in each group. After the treatment, the mice were observed and their survival rates were determined.

2.5. Immunocytometry Assay (IC) and Western Blot Analyses

To determine the expression of viral proteins in the JEV-infected tissues and cells, IC and western blot analyses were performed using anti-E monospecific rabbit sera [15]. In the IC assay, brain tissues and cells were fixed and then incubated with anti-E sera (1:500) for 1 h at 37C. In the second reaction, cells were incubated with FITC-conjugated anti-rabbit IgG goat sera for 1 h at 37C, and then observed by fluorescence microscopy (Olympus, Japan). In the western blot analyses, JEV-proteins were detected using a peroxidase-conjugated secondary antibody against rabbit IgG and observed by the LAS 4000 (Fuji film, Japan).

2.6. IFN Assay

To measure the amount of interferon (IFN) produced in the cell culture fluids and mouse sera, we used as IFN assay kit for the human IFN-β (Hu-IFN-β) and mouse IFN-α (Mo-IFN-α) which were purchased from PBL Biomedical Laboratories. Briefly, the procedure is as follows. The HepG2 cells were transfected with poly (I:C) (Calbiochem) (10 μg/ml), pSilencer (10 μg/well) or pJRi (10 μg/well). DNAs were transfected into the cell with Lipofectamine 2000 (Lipo 2000) reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 5 hr in 0.5 ml medium containing the DNA and Lipo 2000 (10 μl) reagent. After the removal of the trasfection mixture, 1 ml of fresh medium was added and cells were incubated for an additional 12 hr. Cell culture fluids were collected and assayed for Hu-IFN-β production using the enzyme-linked immunosorbent assay (ELISA) kit.

Using the hydrodynamic-transfection method, 2.0 ml of saline containing pJRi, pSilencer, poly(I:C) or pcDNA-FLAG was injected into the tail veins of mice within 5 - 10 second. Blood was collected from the tail vein of each mouse at 12 and 24 hr after the injection. Blood serum was separated by centrifugation and the mouse interferon alpha (Mo-IFN-α) in the serum was measured using the Mo-IFN-α ELISA kit. IFN levels in the cell-culture fluids or blood sera were calculated using a standard curve. However, large deviations were observed between the experimental absorbance values obtained at 450 nm and standard curves, especially at lower Mo-IFN-α levels. The detection limit for this assay was ca. 50 pg/ml for IFN-α in blood sera.

3. Results

3.1. Viral Replication in Mice and Cultured Cells

JEV can replicate well in mouse, but the virulence is different between viral strains. Actually JEV Ishikawa 10 strains isolated in 2010 indicated a weak virulence to mouse in comparison with JaGAr01, as shown in **Figure 1**. In addition, Ishikawa strain expressed viral antigen E protein relatively lower level in mouse neuron (**Figure 2**), and human neuroblastoma IMR32 cells (**Figure 3**), although usually JEV can grow well in various cell lines, ranging from mosquito to human cells.

To examine the effect of shRNA on the JEV replication, we used the virulent strain JaGAr01.

3.2. Inhibitory Effect of pJRi on JEV in Infected Mice

We have already demonstrated efficient antiviral activity of 3 different types of RNAi (JCR, JN3R and JRi) *in vitro*. Using HepG2 and Vero cells, it was shown that JRishRNA efficiently inhibited the replication of JEV [12]. The antiviral activity of JRi, which was produced in the cells transfected with pJRi, was relatively higher than the antiviral activities of JCR and JN3R. Therefore, we considered pJRi as a good candidate antiviral reagent and used it in the animal experiments. To confirm the antiviral activity of pJRi, ICR mice, which were infected with the JEV JaGAr01 strain (5000 PFU), were treated with 50 μg or 100 μg of pJRi/mouse. After the treatment,

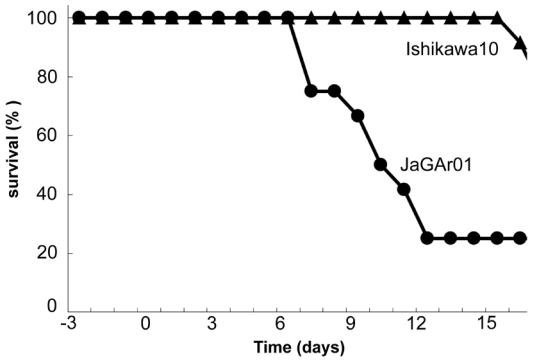

Figure 1. Survival rate of JEV-infected mice. Mice were inoculated i.p. with two kinds of JEV strains, *i.e.* JaGAr01 (-●-) and Ishikawa10 (-▲-), and observed. Each experimental group contained 9 mice.

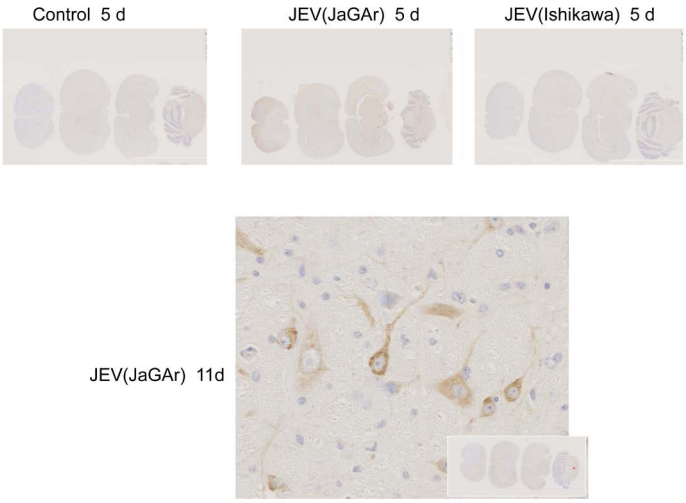

Figure 2. Pathology of JEV-infected mouse brain: Mice were inoculated i.p. with two kinds of JEV strains, *i.e.* JaGAr01 and Ishikawa 10. Mouse brains were taken from JEV-mice and embedded into paraffin after formalin fixing. The brain tissues were immunostained using anti-E.

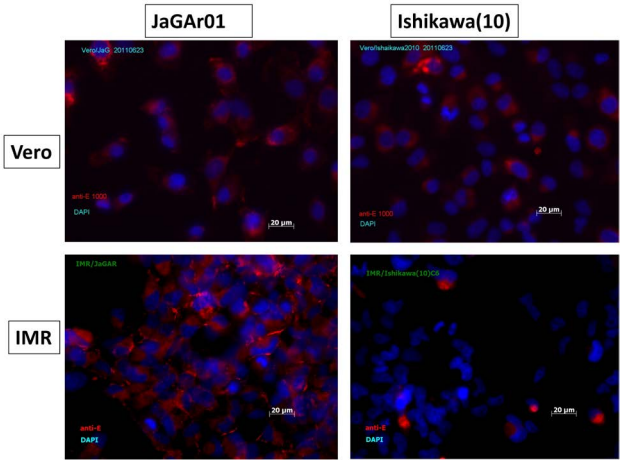

Figure 3. Expression of E protein in JEV-infected cells. Vero and IMR cells were infected with JEVs, JaGAr01 and Ishikawa 10. After 24 hrsp.i., cells were fixed and then immunostained with anti-E.

mice were continuously observed and their survival rates were determined on the day 20 p.i. As shown in **Figure 4**, the survival rates of mice injected with 50 μg and 100 μg pJRi/mouse were 50% and 75%, respectively. These results clearly indicated that pJRi prohibited the viral replication in the mice. The data in **Figure 4** also indicated that the control vector pSilencer (100 μg/mouse) by itself could inhibit JEV replication in mice (survival rate: 50%). One of the reasons for this phenomenon might be the innate immunity induced by the injection of plasmids, including the pSilencer.

Next, we used many mice to confirm whether the plasmid pJRi actually had the ability to protect mice against viral infection. To determine on which day transfection with pJRi is most effective in protecting mice against JEV, we injected the mice with a given plasmid on 3 days before (−3 d), on the same day (0 d), and 3 days after (+3 d) the JEV infection. On the day 20 after the viral infection, the survival rate of control mice infected with JEV was about 10% (**Figure 5(a)** and **Figure 5(b)**). The survival rates of mice injected with 100 μg/mouse pJRi on −3 d, 0 d and +3 d were 22%, 78% and 44%, respectively (**Figure 5(b)**). The control vector pSilencer (100 μg/mouse) could inhibit JEV replication in mice (survival rate: 50%), as previously described (**Figure 4**). Here, only the mice treated with pSilencer on 0 d showed high survival rate and some efficacy to protect the mice against JEV (**Figure 5(a)**). These data indicate that the injection with pJRi on the same day as the viral challenge was most effective in protecting the mice. Our results suggest that JEV replication can be efficiently inhibited by RNAi.

3.3. IFN Response to JEV Infection and Plasmid Injection

JEV replication can be influenced by the innate immunity including IFN. At first, we examined the induction of

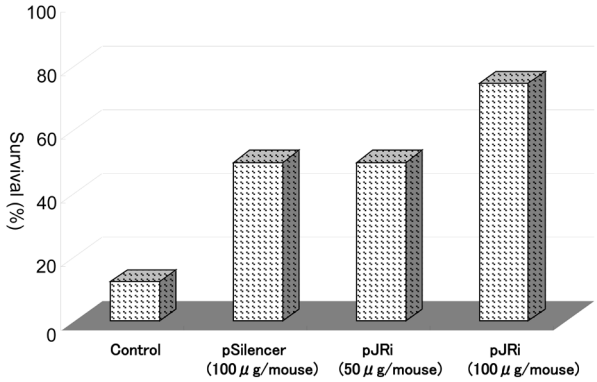

Figure 4. Effect of plasmids on the survival rate of JEV-infected mice. Mice were inoculated i.p. with JEV virulent strain, JaGAr01, and then injected with plasmids (pSilencer and pJRi). After 15 days, survival rate was determined. Each experimental group contained 5 mice.

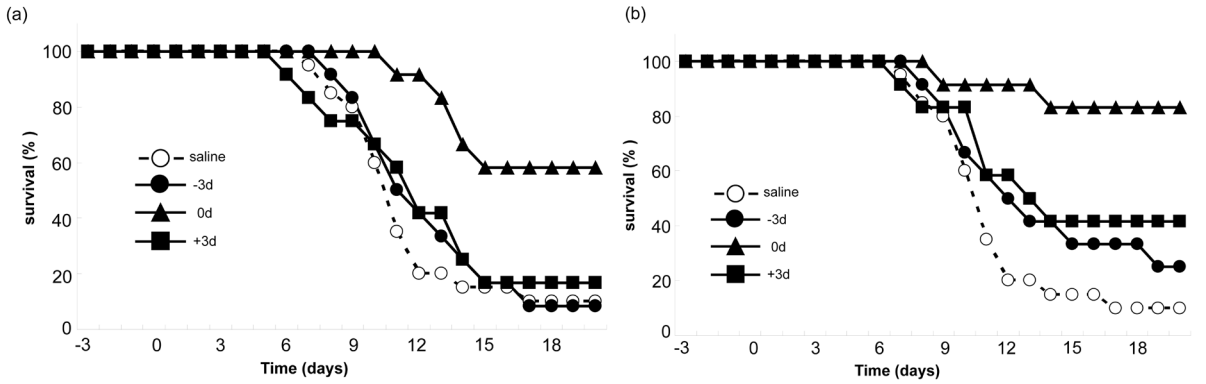

Figure 5. Effect of plasmids on the survival rate of JEV-infected mice. Mice were inoculated i.p. with JEV virulent strain, JaGAr01. Plasmids (100 ug) of pSilencer (a) or pJRi (b) were injected before 3 days (-●-), same day (-▲-) and after 3 days (-■-) the inoculation of JEV. Each experimental group contained 9 mice.

IFN in HepG2 cells transfected with a given plasmid. Hu-IFN-β levels in the cell culture fluids were assayed at 12 hr after transfection with poly (I:C), pSilencer or pJRi. As expected, poly (I:C) strongly influenced the IFN-β production in the cells. In comparison with poly (I:C), effects of plasmids pJRi and pSilencer were not so strong on IFN production, although the effect of pJRi was slightly higher than that of the pSilencer (data not shown).

Next we examined the effect of plasmids on IFN-α level in mice. **Figure 6** shows the influence of plasmid injection on the IFN level in the mouse sera. Clearly, the IFN level in the pJRi injected mouse was 50 - 100 pg/ml at 12 - 24 hr after the treatment, which was lower than that (IFN ca 500 pg/ml) in the JEV infected mouse. In addition, the effect of pJRi on IFN production was not so different from that of the control vector pSilencer. However, another control vector pcDNA-Flag had almost no effect on IFN production (**Figure 6**). These results suggested that the pSilencer plasmid by itself had the ability to induce IFN production in mice.

IFN level in mouse sera seems to be related withthe JEV replication. We examined IFN sensitivity and the relationship between JEV replication and IFN level in cultured cells Vero. As shown in **Figure 7**, JEV JaGAr01 reproduction in Vero cells was clearly suppressed in the presence of IFN-β 15 ng/ml. The expression of JEV protein E was also inhibited by the IFN-β.

4. Discussion

JEV is responsible for over 30,000 annual cases of encephalitis in the world, although there have been less than 10 JE cases each year since 1992 in Japan [4]. In order to avoid future JE outbreaks, it is necessary to carefully monitor changes of viral virulence [4] [16]-[18]. In this study, we confirmed the difference in the virulence and neuro-invasiveness in mouse between two strains JaGAr01 (genotype 3) and Ishikawa 10 (genotype 1). Ishikawa 10 strain was newly isolated from the mosquitoes collected in Ishikawa area in 2010, and its replication or neuro-

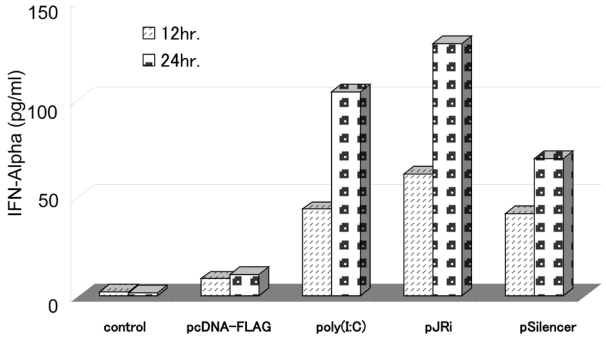

Figure 6. IFN level in mouse sera: Mice were injected with plasmids and poly (I:C). After 12 (light gray) and 24 hrs (dark gray), IFN levels in the sera were examined by the assay kit described in the text.

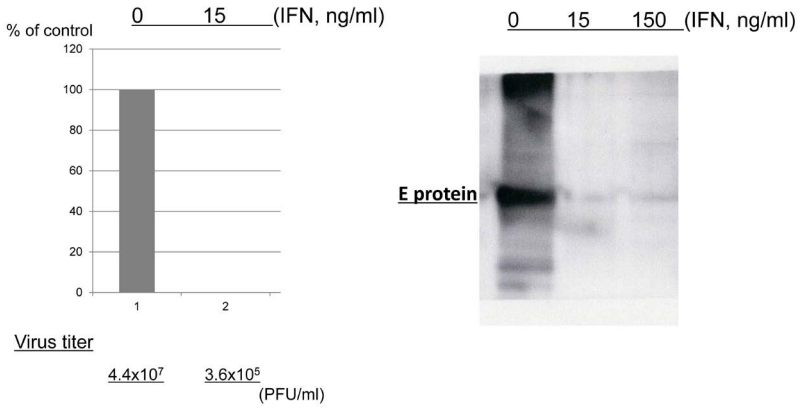

Figure 7. IFN level in mouse sera: Mice were injected with plasmids and poly (I:C). After 12 (light gray) and 24 hrs (dark gray), IFN levels in the sera were examined by the assay kit described in the text.

invasiveness into mouse brain was markedly lower than that of JaGAr01 as shown in the result (**Figures 1-3**). It is obvious that virulence of Ishikawa 10 to mouse was remarkably low level. Growth rate of Ishikawa 10 in the neuroblastoma IMR cells was also lower than that of JaGAr01. These results suggest that the ability of JEV Ishikawa 10 may be weak in the replication or affinity to neuron. The virulence of JEV is different between strains, but we cannot say whether genotype 1 JEVs are weak in the virulence or not, until much more data are collected [17]-[19].

To examine the antiviral effect of shRNA on JEV replication *in vivo*, we used JaGAr01 as the representative virulent JEV strain. Previously we reported that the shRNAJRi indicated its strong inhibitory effect on JEV replication in the cell [12]. Here, against the virulent strain JaGAr01, we found that the shRNAJRi was also effective to inhibit viral replication in mice (**Figure 4**, **Figure 5**).

JRi that corresponded to the prM region of the JEV genome, could bind to JEV RNA, and function as small interfering RNAs (siRNA). It is thought that RNAi is initiated by the RNase III-likeenzyme Dicer. Subsequently, the siRNA is incorporated into the RNA-Induced Silencing Complex (RISC), which uses the siRNA as a guide to target the complementary mRNA for cleavage [10]. Here, it is necessary to consider the possibility of the RNAi effect of shRNAJRi, which could induce small-interfering RNA-mediated antiviral silencing [20]-[24]. JRi is incorporated into the RISC, and should be effective in digesting the JEV genomic RNA. These results are related to the suppression of other flavivirus infection [25] [26].

We expected that the inhibitory effect of pJRi would continue for several days, since several reports suggested that the life spans of shRNA expressing vectors were long. However, as shown in **Figure 5**, the result indicated that only injecting the mice with pJRi on the same day (0 d) as the viral challenge was the most effective in protecting the mice against JEV infection. Our shRNAJRi, although still expressed in the mouse tissue, may have been diluted in the animal experiments. On the basis of these results, it would be necessary to create a new system to keep a sustained inhibitory effect of pJRi against JEV infection (e.g. blood cells are good candidates to carry the shRNA expressing plasmid pJRi).

Interestingly, inhibitory effect on JEV replication in the mice was observed in the injection of not only pJRi but also the empty vector pSilencer (**Figure 4**, **Figure 5**). There is a possibility that injection of plasmids into the mice, including pJRi, might influence their innate immune systems. Such an IFN inducing activity may depend on the CG content of the plasmid DNA. To examine the effects of plasmids on the immune response mechanism, we measured the IFN level in mice. PolyI:C, pJRi and pSilencer had the activity to induce the IFN level. However, even though pSilencer and pJRi have IFN inducing activity, this is still not enough to explain the inhibitory effect of pJRi on JEV infection in mice. As revealed by our data, the survival rate of mice treated with pJRi (100 μg/mouse, 0 d) was higher than that treated with pSilencer (100 μg/mouse, 0 d), suggesting that the JRi could inhibit JEV infection or reproduction *in vivo* with high potency. At the same time, we confirmed that actually JEV replication in the cultured cells was strongly inhibited in the presence of IFN (**Figure 7**), as several reports described previously [27]-[31]. It suggests that JEV is sensitive against IFN, that is, the situation of JEV replication is closely related with the level of IFN in the tissues [30] [31].

Ourdatamean that pJRi has the remarkable inhibitory activity against JEV infection *in vivo*, and could be used as an antiviral drug to treat JEV infection. On the other hand, we should be careful about the evolution and outbreak of JEV and other flaviviruses in the future [4] [32] [33].

Taken together, shRNAJRi is effective to inhibit JEV replication through the RNAi action and the additive activity inducing IFN. However, no conclusion about the mechanism of antiviral activity of pJRi can be drawn from above described results, and further experiments are warranted to understand the underlying mechanism.

Acknowledgements

This study was supported in part by the Project Research from High Technology Center (H2011-10) of Kanazawa Medical University and the Science Research Promotion Fund of the Promotion and Mutual Aid Corporation for Private Schools of Japan, and grants (Research (C), 18590455, 21610024 and 25430119) from the Ministry of Education, Culture, Sports, Science and Technology of Japan

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