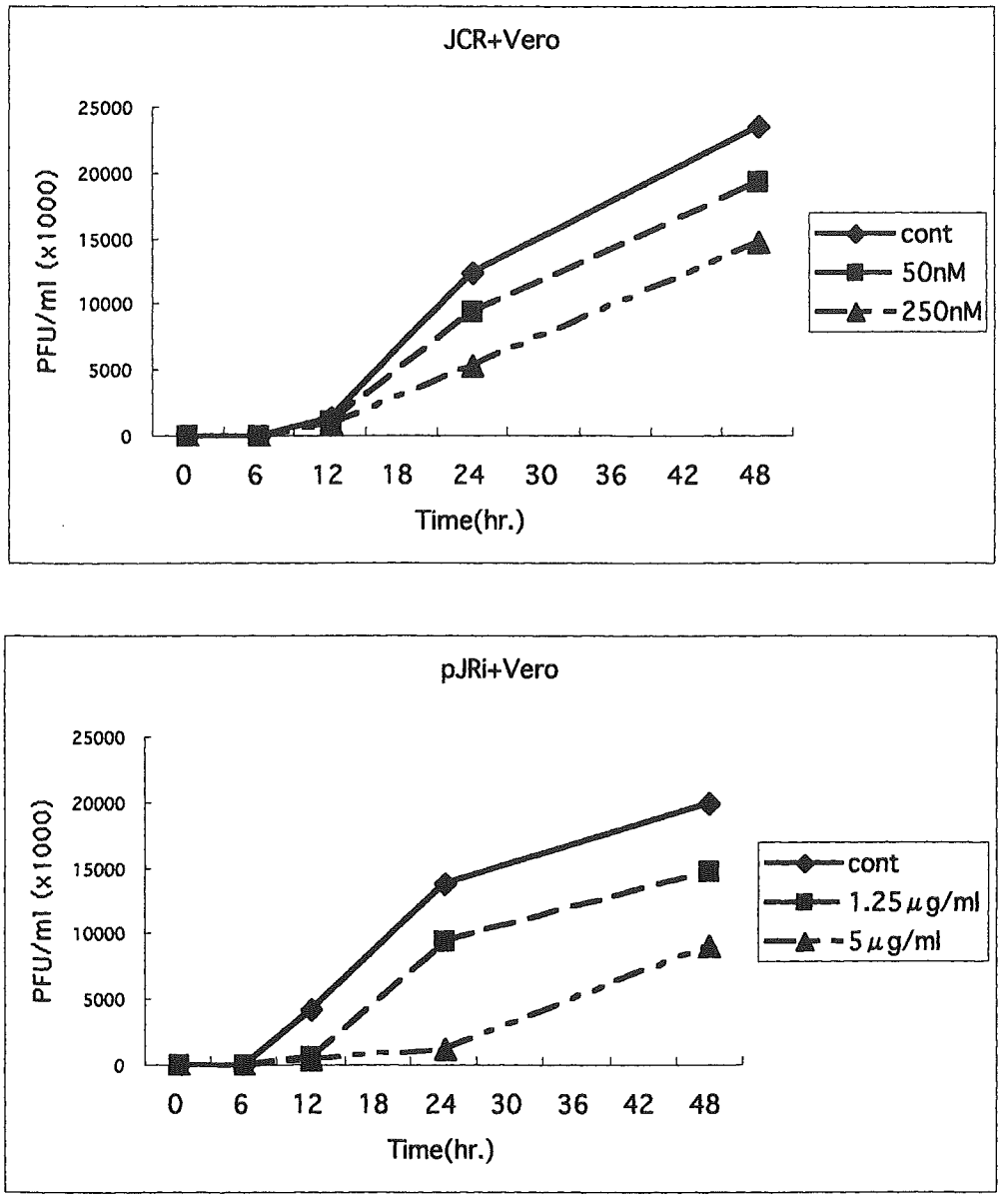


Fig. 3. Inhibitory effect of JCR and pJRi on JEV replication with the lapse of time in Vero cells. Vero cells were transfected with JCR (50 and 250 nM) and pJRi (1.25 and 5 μg/ml). These cells were transfected with RNAi and infected with JEV, as described in the legend to Fig. 2. The cells were cultured for an additional 0, 6, 12, 24 and 48 hr. JEV titer in the culture fluids was assayed by the plaque method using BHK cells.

ity of JCR was relatively higher than that of JN3R in Vero cells (Fig. 4a). However, the inhibitory effect of both RNAis on viral reproduction in HepG2 cells was almost the same (Fig. 4b). The inhibitory effect of pJRi which could produce the stem-loop type RNAi was also observed to be similar between Vero and HepG2 cells (Fig. 5). When the RNAis were transfected into the cells after JEV infection, the inhibitory activity of RNAi was also observed but was not so high as described above (data not shown).

Analysis of Viral Envelope (E) Proteins by Western Blot

We examined the E protein (53 kDa) expressed in JEV-infected Vero and HepG2 cells by western blot analysis. The expression level of E protein in JEV-infected cells was dose-dependently decreased by RNAi treatment (Figs. 6 and 7). The relative amounts of E protein were calculated by the NIH image program. JCR and JN3R reduced E protein expression in JEV-infected cells. E protein levels in JCR- and JN3R-Vero cells were 30.1 and 37.9% of control, as shown in Fig. 6a. The inhibitory effect of JCR on viral protein expres-

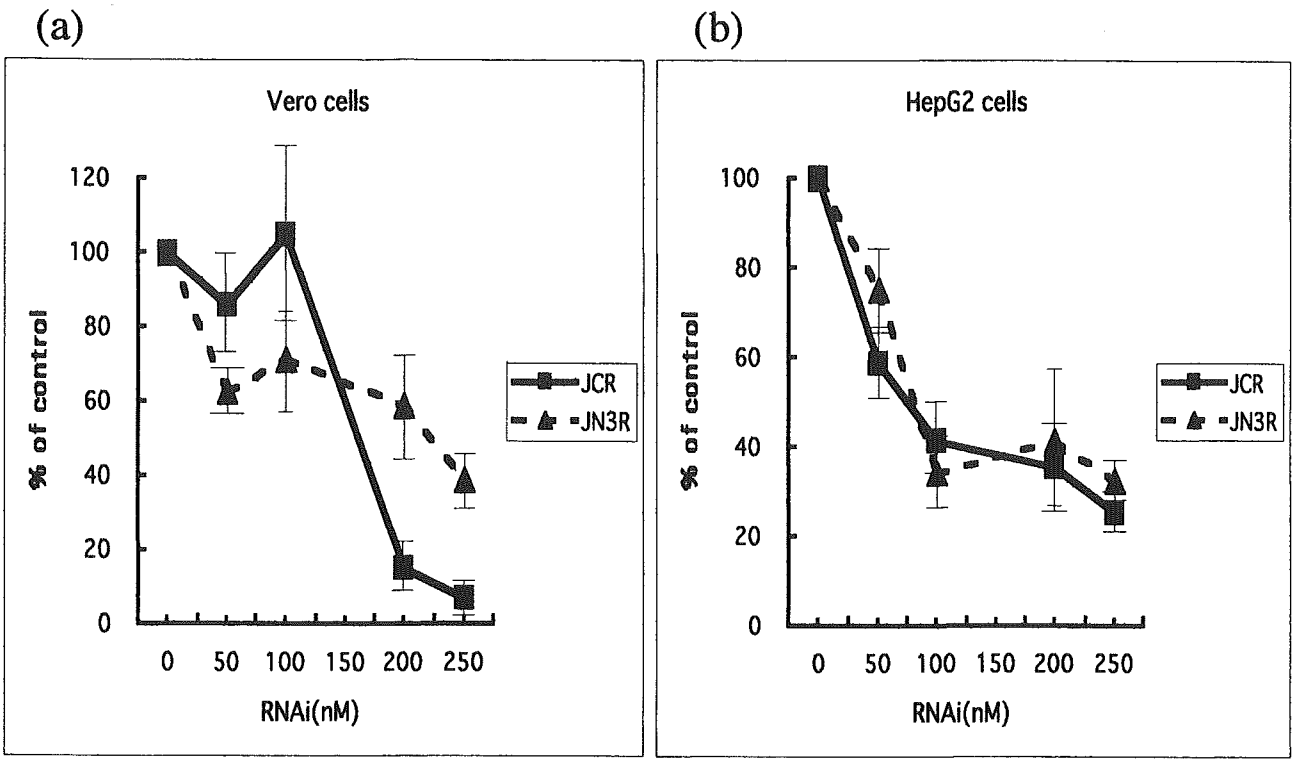


Fig. 4. Inhibitory effect of JCR and JN3R on JEV replication. Vero (a) and HepG2 (b) cells were transfected with JCR and JN3R (50, 100, 200 or 250 nM), respectively. The cells were then infected with JEV. After virus infection, 2 ml of fresh growth medium was added and cells were cultured for an additional 24 hr. JEV titer in the culture fluids was assayed by the plaque method using BHK cells.

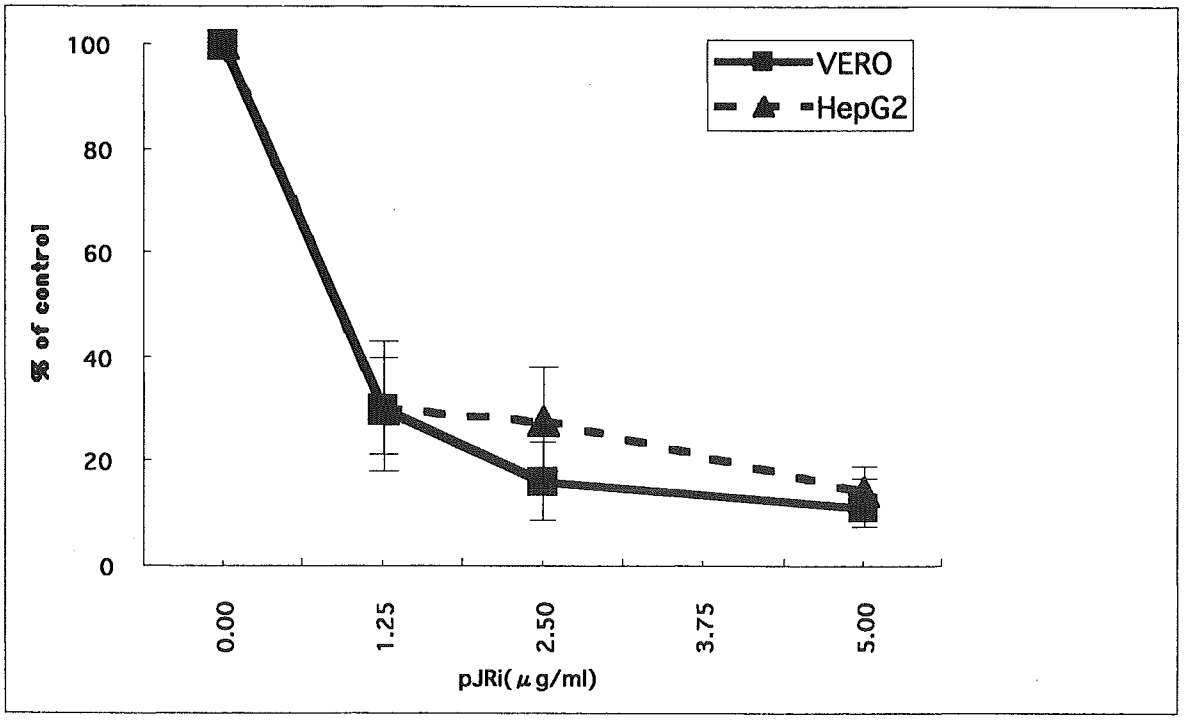
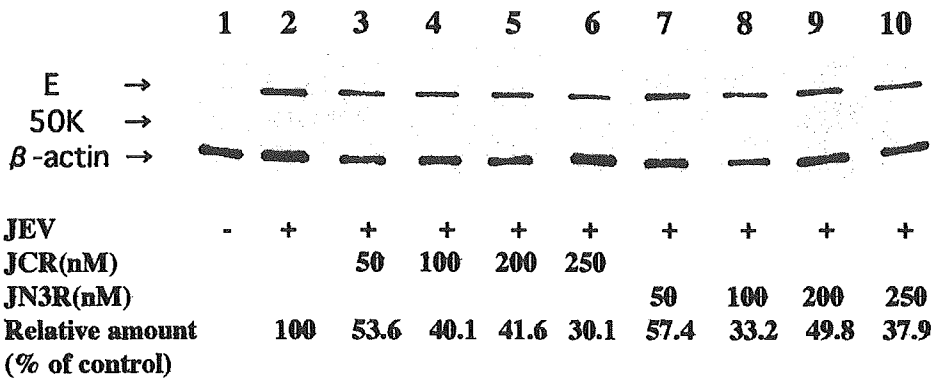


Fig. 5. Inhibitory effect of pJRi on JEV replication. Vero and HepG2 cells were transfected with pJRi (1.25, 2.5 or 5 μg/ml). At 24 hr after RNAi transfection, the cells were infected with JEV and cultured for additional 24 hr, as described in the legend to Fig. 2. JEV titer in the culture fluids was assayed by the plaque method using BHK cells.

(a)



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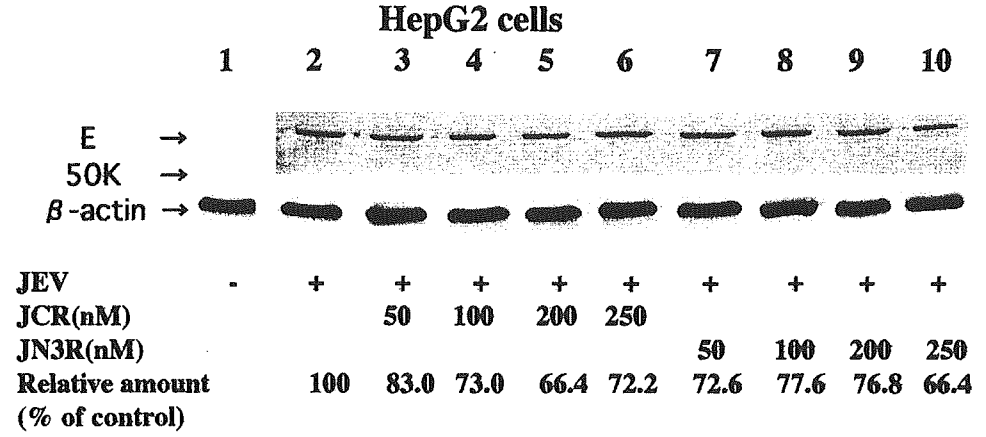

Fig. 6. Detection of JEV-specific E protein by western blot. Vero (a) and HepG2 (b) cells were transfected with JCR or JN3R before the infection with JEV. Protein samples were subjected to SDS-PAGE transferred to a PVDF membrane and reacted with anti-E. β-Actin served as a loading control. The relative amounts of JEV specific E protein were calculated by the NIH image program.

sion was slightly higher than that of JN3R (Fig. 6). The transfection of pJRi also inhibited E protein expression in JEV-infected cells (Fig. 7). The transfection of 5 μg/ml pJRi reduced E protein expression to 36.5 and 24.7% of control level in Vero and HepG2 cells, respectively.

Protection by pJRi-Injection against JEV Infection

In order to examine the effect of RNAi, an *in vivo* assay using ICR mice was carried out. In the *in vitro* assay using the cultured cells, the data indicated that pJRi effectively inhibited the expression of E protein in JEV-infected cells (Fig. 7). The effect of pJRi was more remarkable than those of JCR and JN3R (Figs. 4–7). Therefore, we used pJRi in the animal experiment. After the inoculation of viruses at 5×10^3 PFU, pJRi at 1.0 or 5.0 μg/g was injected into mice i.p. JEV-infected control mice (n=5) died within 15 days. Twenty percent of mice transfected with empty vector, pSilencer, survived at 15 days. On the other hand, pJRi (1.0 or 5.0 μg/g)-medicated mice survived at 40 or 80% at 15 days (Fig. 8). Figure 9 shows the body weight of the mice. The body weight of mice increased to ca. 26 g at 6 days after JEV infection, but the weights of control (JEV+) and empty vector transfected mice markedly decreased and they were 19.7 g and 22.6 g at 12 days p.i., respectively. The weight of mice medicated with pJRi 5.0 μg/g, however, still increased to 27.0 g at 9

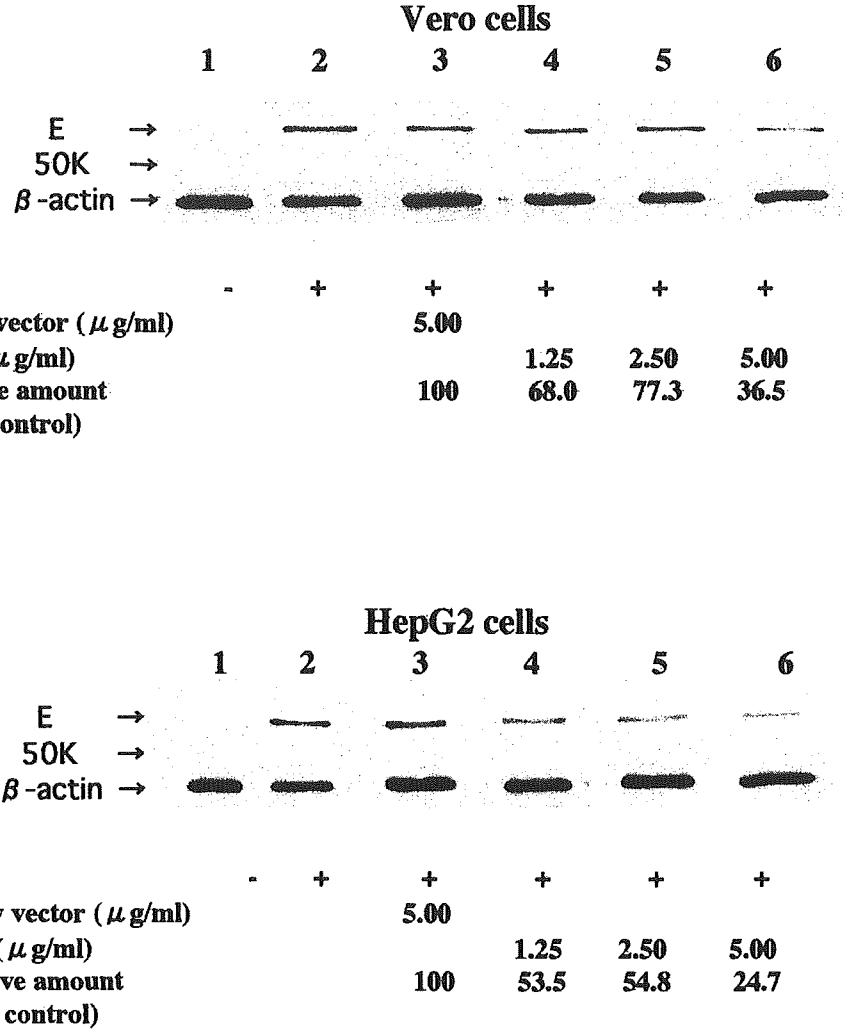

Fig. 7. Detection of JEV-specific E protein by western blot. Vero and HepG2 cells were transfected with pJRi before the infection with JEV. Protein samples were subjected to SDS-PAGE, transferred to a PVDF membrane and reacted with anti-E. The relative amounts of JEV-specific E protein were calculated by the NIH image program.

days p.i., as shown in Fig. 9. The data indicate that pJRi had an inhibitory effect on JEV reproduction and no side effects in mice.

Discussion

In this paper, we made use of 3 kinds of RNAi, i.e. JCR, JN3R and JRi, corresponding to the different parts of the JEV genes C, NS3 and M. C and M are JEV structural proteins and NS3 is a JEV protease domain (17). The structure of JCR and JN3R consists of double-stranded 21 base RNA/DNA chimera (duplex RNA type), and pJRi expression resulted in stem-loop RNA structures in the cells (Fig. 1). As shown in the results, the level of JEV yield in culture fluid was decreased by the RNAi treatment. The level of JEV-specific E proteins estimated by western blot analysis was also reduced by the RNAi treatment.

The RNAi expression process, in general, can be divided into 3 steps: first, dsRNA cleavage by Dicer and the generating siRNAs: second, incorporation of siRNAs into RISC; third, recognition, cleavage, and destruction of the target mRNA by siRNA-RISC (4, 15, 18, 20, 21). RNAis of the duplex RNA type commence to RNAi expression in the second process (18, 20, 21). RNAi is a mechanism that is involved in epigenetic regulation of gene expression, and then it is used to knock down the translation. However, it is unknown whether a similar antiviral mechanism also operates in mammalian cells (8, 12). Because RNAi relies on nucleotide interactions, it can be tailored to almost any gene, whether cellular or viral, and the expression can

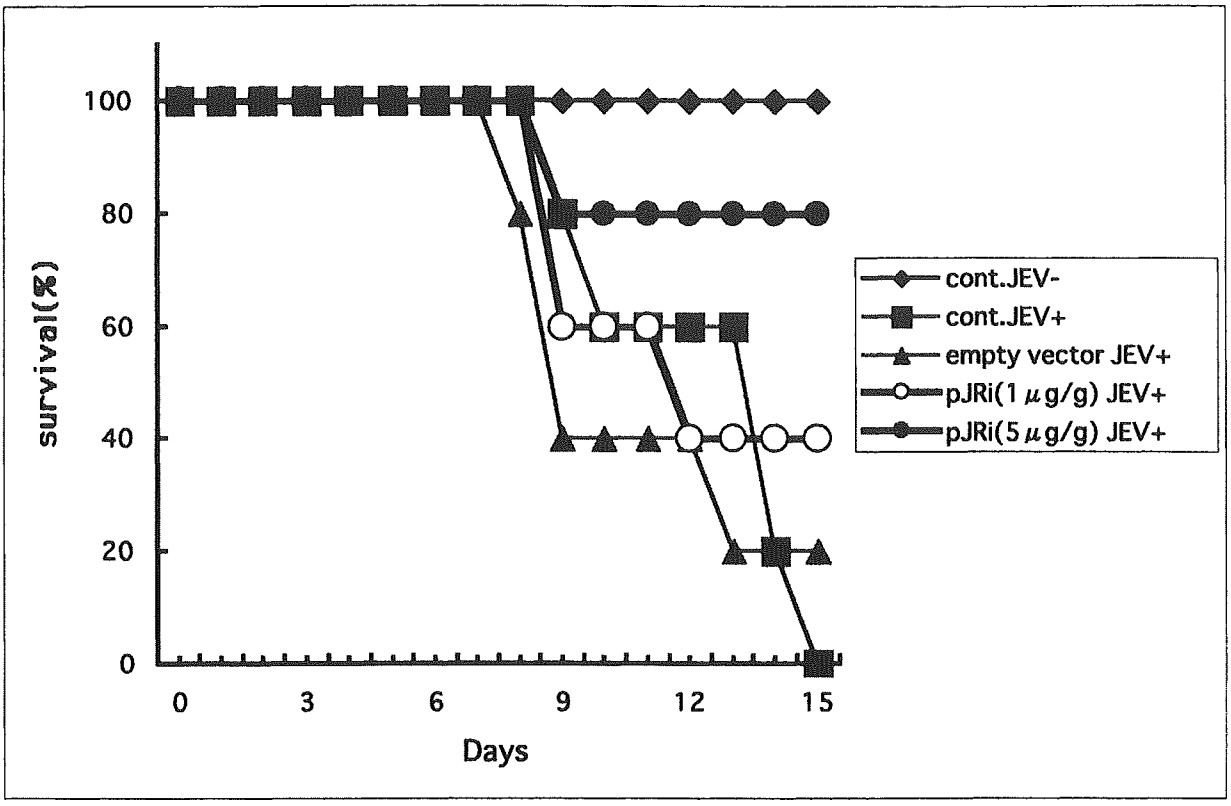

Fig. 8. Survival of JEV-infected mice inoculated with pJRi. ICR mice were injected i.p. with 1.0 or 5.0 μg/g of pJRi in 0.2 ml of saline as soon as the mice were infected with JEV (5×10^3 PFU) per 0.2 ml of saline i.p.

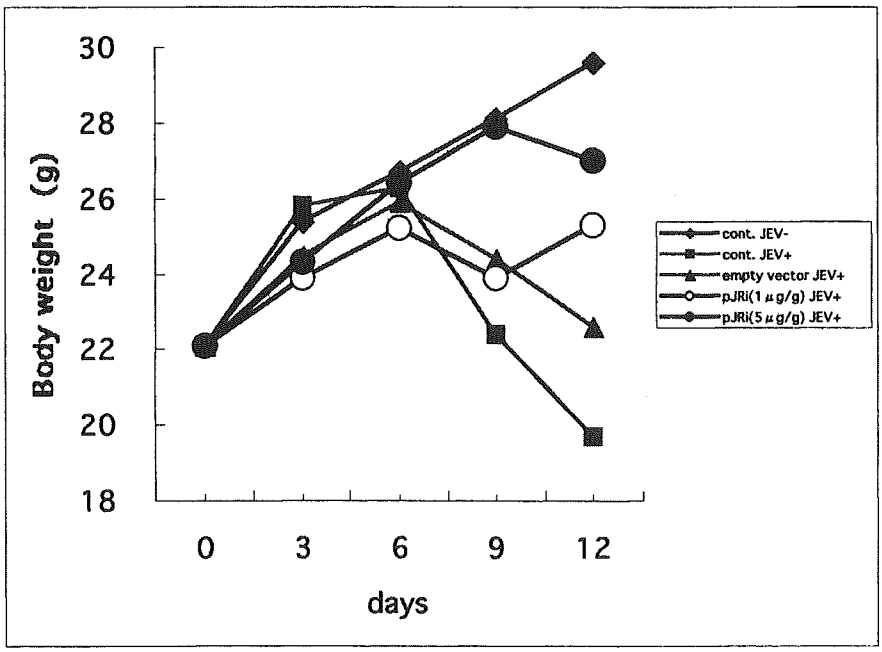

Fig. 9. Body weight of JEV-infected mice inoculated with pJRi. ICR mice were injected i.p. with pJRi after the mice were infected with JEV (5×10^3 PFU), as described in the legend to Fig. 8. Mice were checked at intervals of 3 days.

be knocked down without actual destruction (1, 3, 5, 12–14, 19). Here we indicated that the inhibitory activity of JCR, JN3R and JRi on JEV replication was remarkable. The virus yields in Vero cells were 7.2±3% ($P<0.05$) (JCR) and 39.0±9% ($P<0.05$) (JN3R) of control level by RNAi treatment at 250 nM

(Fig. 4a). The inhibitory activity of JCR was relatively higher than that of JN3R. The decrease of virus yield in RNAi-treated cells seems to be a phenomenon which is in agreement with reduction of the amounts of JEV-specific proteins. The expression of E protein in JEV-infected cells decreased with RNAi treatment (Figs. 6 and 7). In the treatment with duplex RNA-type RNAi (JCR and JN3R), E protein level in Vero cells decreased to 30.1 (JCR) and 37.9% (JN3R) of control level (Fig. 6a). Under similar conditions, the E protein level in HepG2 cells decreased to 72.2 (JCR) and 66.4% (JN3R) of control level. That is, the level of E protein content in Vero cells was much less than that in HepG2 cells after JCR and JN3R treatment. From the virus replication data in HepG2 cells, inhibitory activity of JCR and JN3R was also clearly observed, and inhibition by JCR was a bit higher than that by JN3R (Fig. 4). The inhibitory activity of pJRi on JEV replication was similar between Vero and HepG2 cells (Fig. 5). These facts are the consequence of RNAi nucleotide sequences, since they are related to the region of the JEV genome. In addition, the effects of RNAi seem to be influenced by the condition of cells. The difference in the inhibitory effect of RNAi between Vero and HepG2 cells may be based on the difference in cellular factors. The RNAi expression step in RISC assembly is an unexpected source of asymmetry for both siRNA function and biogenesis.

The inhibitory effect on JEV reproduction by pJRi was clearly observed in both Vero and HepG2 cells, as described. The relative amount of E protein decreased to 36.5 and 24.7% of control level in Vero and HepG2 cells treated with the pJRi at 5 μg/ml (Fig. 7). The results in the *in vitro* RNAi experiment show the efficacy against JEV replication *in vivo*. In the case of *in vivo*, survival assay, mice began to die within 8 or 9 days after JEV infection. The survival rate of pJRi (1.0 or 5.0 μg/g)-medicated mice was 40 or 80% at 15 days (Fig. 8). On the other hand, the survival rate of the mice transfected with the control vector was 20% at 15 days. The body weight of the mice, including empty vector-injected mice, decreased within 6 days after JEV infection, but that of pJRi (5.0 μg/g)-medicated mice increased 26.4 to 27.0 g, the same as the control (JEV−) (Fig. 9). These results agree with the survival rates and suggest that the RNAis could be used as antiviral drugs in medical treatment against JEV infection.

Taken together, the following conclusions were reached. The RNAis have highly potent inhibitory activity against JEV replication *in vitro* and *in vivo*. It is possible that RNAis will be able to be used as inhibitors of flavivirus replication including dengue, yellow fever and West Nile viruses. However, for the development of a useful antiviral drug, further experiments using RNAi are necessary.

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References

1) Agami, R. 2002. RNAi and related mechanisms and their potential use for therapy. Curr. Opin. Chem. Biol. **6**: 829–834.
2) Centers for Disease Control and Prevention (CDC) Home-Page (http://www.cdc.gov/)
3) Cerutti, H. 2003. RNA interference: traveling in the cell and gaining function? Trends Genet. **19**: 39–46.
4) Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K., and Saigo, K. 2003. Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr. Biol. **13**: 41–46.
5) Dykxhoorn, D.M., Novina, C.D., and Sharp, P.A. 2003. Killing the messenger: short RNAs that silence gene expression. Nat. Rev. Mol. Cell Biol. **4**: 457–467.
6) Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15**: 188–200.
7) Glenn, R., Arash, G., and Charles, M.R. 2003. Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc. Natl. Acad. Sci. U.S.A. **100**: 235–240.
8) Gordon, G.C. 2002. Silencing viruses with RNA. Nature **418**: 379–380.
9) Infectious Disease Surveillance Center (IDSC) Home-Page (http://idsc.nih.go.jp/index-j.html)
10) Kameoka, M., Nukuzuma, S., Itaya, A., Tanaka, Y., Ota, K., Ikuta, K., and Yoshihara, K. 2004. RNA interference directed against Poly (ADP-Ribose) polymerase 1 efficiently suppresses human immunodeficiency virus type1 replication in human cells. J. Virol. **78**: 8931–8934.
11) Kapadia, S.B., Brideau-Andersen, A., and Chisari, F.V. 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc. Natl. Acad. Sci. U.S.A. **100**: 2014–2018.
12) Kawasaki, H., and Taira, K. 2003. Short hairpin type of dsRNAs that are controlled by $tRNA^{Val}$ promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res. **31**: 700–707.
13) Lavery, K.S., and King, T.H. 2003. Antisense and RNAi: powerful tools in drug target discovery and validation. Curr. Opin. Drug Discov. Devel. **6**: 561–569.
14) Li, H., Li, W.X., and Ding, S.W. 2002. Induction and suppression of RNA silencing by an animal virus. Science **296**: 1319–1321.
15) Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R.,

and Tuschl, T. 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110**: 563–574.
16) National Institute of Infectious Diseases (NIID) Home-Page (http://www.nih.go.jp/niid/index-e.html)
17) Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L., and Strauss, J.H. 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science **229**: 726–735.
18) Schwarz, S.D., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, D.P. 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell **115**: 199–208.
19) Shi, Y. 2003. Mammalian RNAi for the masses. Trends Genet. **19**: 9–12.
20) Shuey, D.J., McCallus, D.E., and Giordano, T. 2002. RNAi: gene-silencing in therapeutic intervention. Drug Discov. Today **7**: 1040–1046.
21) Silva, J.M., Hammond, S.M., and Hannon, G.J. 2002. RNA interference: a promising approach to antiviral therapy? Trends Mol. Med. **8**: 505–508.
22) Takegami, T., and Hotta, S. 1990. Synthesis and localization of Japanese encephalitis virus RNAs in the infected cells. Microbiol. Immunol. **34**: 849–857.
23) Takegami, T. 2003. Japanese encephalitis. Virus **53**: 25–30 (in Japanese).

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Analysis of yearly changes in levels of antibodies to Japanese encephalitis virus nonstructural 1 protein in racehorses in central Japan shows high levels of natural virus activity still exist

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Abstract

Recent reductions in numbers of human and equine Japanese encephalitis (JE) cases in Japan have seen calls to end JE vaccination. Here, we analyzed yearly variations of natural JE virus activity, using sera collected serially in 1998–2003 from racehorses residing in Ibaraki and Shiga prefectures, both located in central Japan. A total of 208 sera from 24 individuals in Ibaraki and 259 from 27 in Shiga were examined for antibodies to JE virus nonstructural 1 (NS1) protein, a marker of natural infection. The natural infection rate in epizootic seasons, which was determined by a significant increase in NS1 antibody level, was 4.2–26.7% in Ibaraki and 0–41.7% in Shiga, indicating that high levels of JE virus activity still existed in central Japan.

Keywords: Japanese encephalitis; Horse; Natural infection

1. Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that can be fatal in humans and equines [1,2]. The virus is distributed widely in Asia, and in parts of Oceania, causing 50,000 human cases with 10,000 deaths every year. Swine are an effective amplifier in a paradomestic environment and have been considered as the major producer of JEV for transmission to humans and equines [3,4]. The majority of humans and horses infected by bites from infectious mosquitoes result in subclinical infections, but some develop neurological diseases [5,6]. An inactivated Japanese encephalitis (JE) vaccine has contributed to the control of human and equine JE in some countries [7,8].

In Japan, following the introduction of the inactivated JE vaccine, the annual number of JE cases has been dramatically reduced from the order of thousands to less than ten in each of humans [9] and equines [10,11]. Simultaneously, the number of *Culex tritaeniorhynchus*, a major vector of JEV [12], has been decreased by the use of insecticides and improved irrigation schemes for rice cultivation; this decrease is considered to have reduced the opportunity for humans/horses to acquire JEV infections [4,7]. Against this background, questions have been posed whether the reduction in JE cases is the result of vaccination or the decreased number of mosquitoes. Further, recently JE cases have become so few that they can be compared to the number of vaccinees suffering from severe neurologic side effects from the inactivated JE vaccine, like acute disseminated encephalomyelitis [13–15], raising arguments about the necessity to continue vaccinations for humans.

A national JE surveillance program has been conducted to monitor natural JEV activity every epidemic/epizootic season since 1965 in Japan [9]. In this program, 5–8-month old slaughtered pigs, usually 10–20 in number, have been examined for hemagglutination-inhibiting (HAI) antibodies; this is usually performed six to eight times in one season in

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each prefecture. JEV infection of swine populations has been shown every summer across the whole of Japan except for the non-endemic northern areas. However, many pig farms have now been moved far from equine and human residential areas. Further, with the hygienic improvements that have been made to pig farms, the contact between swine and mosquitoes has been considered infrequent [7]. Under these conditions, it is generally believed that a surveillance system that uses only a limited number of pigs cannot always be relied on to estimate current JEV activity around humans and horses.

Surveys of natural JEV infections in humans and equines are considered more straightforward to address the issue concerning the importance of JE vaccines. However, conventional serologic methods, such as neutralization and HAI tests, cannot be used to obtain natural infection rates among vaccinated populations, since these methods do not differentiate antibodies induced by natural infection from those induced by vaccination. Therefore, we have established a method to detect antibodies to nonstructural 1 (NS1) protein of JEV [16]. NS1 antibodies are only induced by natural infection, not by vaccination with purified inactivated JE vaccine; thereby, they constitute a marker of natural infection among vaccinated populations. Our preliminary surveys using sera collected at selected locations in Japan before 2000 showed relatively high NS1 antibody prevalences in humans (10–20% [16]) and equines (15–73% [17]).

The present study was designed to analyze the time course of NS1 antibody levels in individual horses and the yearly variation of natural JEV activity. For this purpose, we used sera collected serially over 3–5 years from individual racehorses residing at two training centers in Ibaraki and Shiga prefectures, located in east-central and middle-central Japan, respectively; areas that have shown moderate to high seropositivities in swine as reported by the national JE surveillance program (Fig. 1). Different from sera collected at one point in time, serial sera provide critical information about the timing of acquiring natural infections and the duration of NS1 antibody responses to the infection. Results of the present study indicated that horses in central Japan were still frequently exposed to natural JEV infections up to 2003.

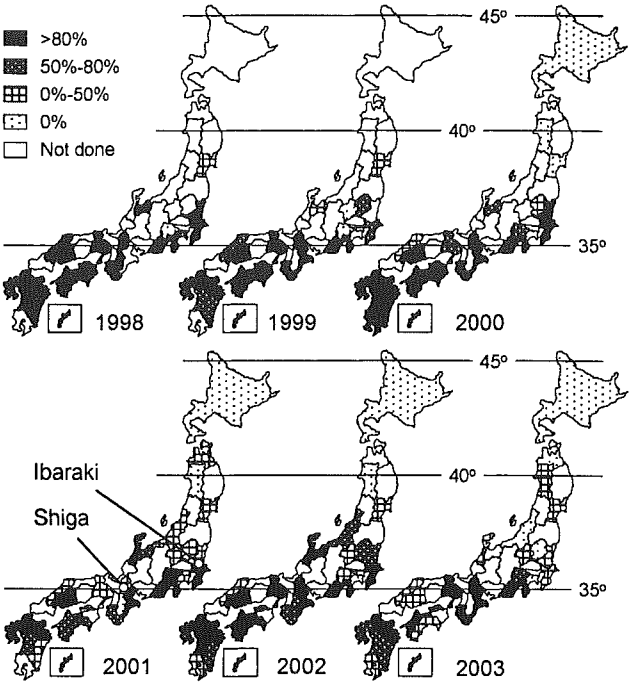

Fig. 1. Maps of Japan indicating locations of Ibaraki and Shiga prefectures (lower left) and seropositivity of the swine population in each prefecture from 1998 to 2003 as reported by the national JE surveillance program [9,20]. Seropositivity of swine is expressed semiquantitatively in the figure. Note that some prefectures did not conduct a surveillance program, such areas are kept open. The island depicted in the square is Okinawa Prefecture, the southernmost prefecture in Japan. Solid lines indicate latitude expressed in degrees north.

2. Materials and methods

2.1. Serum samples

Sera were collected from thoroughbred racehorses residing at the Miho training center in Ibaraki Prefecture and the Rittoh training center in Shiga Prefecture, both located in central Japan (Fig. 1). Racehorses are subjected to blood examinations for equine infectious anemia, primarily every May and November. The training centers used for this study are the two largest in Japan. After being examined, the sera, for which there was an exact record of the vaccination history and blood collection date, were stored at −30 °C for a relatively long period. A total of 208 sera serially collected from 24 horses in Ibaraki between May 1999 and November 2003 and 259 sera from 27 horses in Shiga collected between May 1998 and November 2003 were available from these centers and were used for the present study. Since the JE epizootic season usually starts from July and ends in October, sera collected in May and November are basically representative of the status before and after the epizootic period, respectively.

Ages of these racehorses at the start of the survey period ranged from 3 to 7 years, average 4.5, in the Ibaraki population and from 2 to 6 years, average 3.3, in the Shiga population (see Figs. 3 and 4). Males were predominant in both the Ibaraki (22/24, 92%) and Shiga (25/27, 93%) populations. All horses were vaccinated with inactivated JE vaccine two to four times every year, basically prior to the epizootic period.

2.2. ELISA for quantifying NS1 antibodies in horse sera

Antibodies to JEV NS1 were quantified by a reproducible ELISA using captured NS1 antigen, as previously reported [18]. Briefly, microplates sensitized with rabbit anti-NS1 hyperimmune sera at a 1:10,000 dilution were incubated with 3 ng/well of NS1 antigen obtained from culture fluids of cells stably producing an extracellular form of NS1 antigen. The plates were then incubated serially with test sera at a 1:100 dilution, alkaline phosphatase-conjugated rabbit anti-

horse IgG at a 1:1000 dilution, and *p*-nitrophenyl phosphate at 1 mg/ml. Tests were done in duplicate and absorbances obtained from the two wells were averaged.

To eliminate nonspecific reactions, a non-sensitized control plate was run in parallel and the difference in absorbances from antigen-sensitized wells was obtained. When the subtraction provided a minus value, we assigned 0.000 to the result. To minimize interplate variations, absorbances obtained with test samples were adjusted with the value for the positive control as 0.5. The adjusted absorbances were expressed as ELISA values, which were considered as specific NS1 antibody levels. Sera with ELISA values of ≥0.122 were determined to be positive for NS1 antibodies. The cut-off value (0.122) was determined by calculating a confidence limit from the mean and standard deviation at the probability level of 0.01% based on NS1 antibody levels obtained with 46 sera from yearlings born and kept in a northern non-endemic area (Hokkaido). NS1 antibody levels in vaccinated yearlings kept in Hokkaido did not exceed 0.122, indicating that NS1 antibody level was not affected by vaccination [18].

For analysis of significant differences of NS1 antibody levels between the first and second samples in paired sera with half-year intervals, differences over the 30% of the first antibody levels were regarded as significant differences from the corresponding second antibody levels, excepting samples with a first antibody level of less than 0.333 in which differences of 0.1 or more were considered significant. Seroconversion was defined as a significant increase of ELISA values from <0.122 (negative) to ≥ 0.122 (positive) in a half-year period.

2.3. HAI test

HAI tests were performed as previously described [19], except for the use of a commercial JEV antigen (Denka Seiken, Tokyo, Japan).

2.4. Statistical analysis

Significance of differences in antibody prevalences or percentages of individuals showing increases/decreases in NS1 antibody level was evaluated by the chi-square test with the Yates' correction factor. Significance of differences in mean antibody levels was evaluated by the Student's *t*-test. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. Prevalences of NS1 antibodies and distributions of NS1 antibody levels

To overview a rough situation of natural JEV infections in the present racehorse populations, the prevalence of NS1 antibodies was obtained for each year (Fig. 2). NS1 antibody prevalences were mostly constant during the study period in each of the populations with averages of 60.6% (126/208) in Ibaraki and 52.5% (136/259) in Shiga, without significant difference between them ($P > 0.05$). The lowest prevalence among the Ibaraki population was shown in May 2003 and the highest prevalence was among the Shiga population in November 1998, but these values were not significantly different from the mean prevalence in each corresponding population ($P > 0.05$).

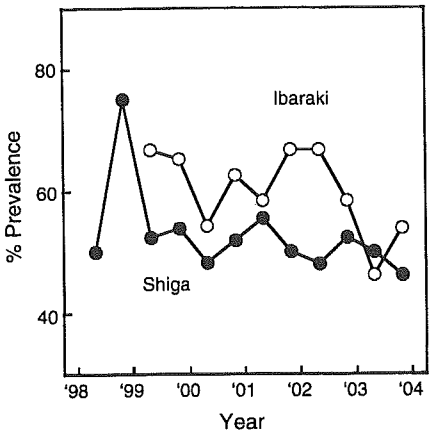

Fig. 2. NS1 antibody prevalences among racehorses in Ibaraki between 1999 and 2003 (open circle) and Shiga between 1998 and 2003 (filled circle).

NS1 antibody levels were distributed from 0.000 to 1.485 in the Ibaraki population and similarly from 0.000 to 1.589 in the Shiga population (data not shown). The mean NS1 antibody levels of positive sera were not significantly different between the populations in each year ($P > 0.05$).

3.2. Time courses of NS1 antibody levels in individual horses and their relation to those of HAI antibody levels

Levels of NS1 antibodies and HAI antibodies obtained in the sera serially collected in Ibaraki and Shiga are shown in Figs. 3 and 4, respectively. Various time courses of NS1 antibody levels indicated individual differences both in the opportunity to acquire natural infections and in the response to the infection. HAI antibody levels were affected by vaccination and sometimes natural infections. An increase in NS1 antibody level was accompanied by a significant (≥4-fold) increase in HAI antibody level in 9 of 19 (47.4%) individuals that seroconverted in both Ibaraki and Shiga populations. Although all horses had been vaccinated, detectable levels of HAI antibodies were not developed in some individuals at the start of the survey period (Figs. 3 and 4: for instance, Ib#3M, Sh#2M) or during some years (Ib#2M, Ib#13M), indicating that the antibody responses to vaccination also varied according to individuals.

3.3. Increases and decreases in NS1 antibody levels during epizootic or non-epizootic seasons

To analyze time courses of NS1 antibody levels, sera were divided into a unit of paired sera at half-year intervals and the

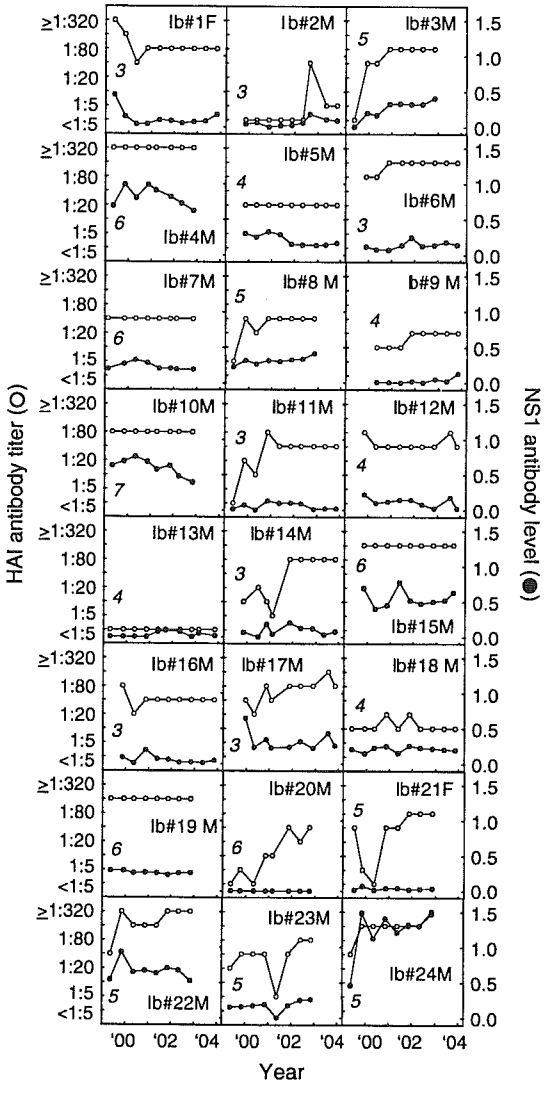

Fig. 3. Time courses of NS1 antibody (filled circle) and HAI antibody (open circle) levels in 24 individual horses in Ibaraki Prefecture. The label in each panel indicates the horse number and gender: for instance, the sample "Ib#1F" was collected from a female horse numbered 1 in the Ibaraki population. Italic numerals indicate the age (year) of horses at the start of serum collection.

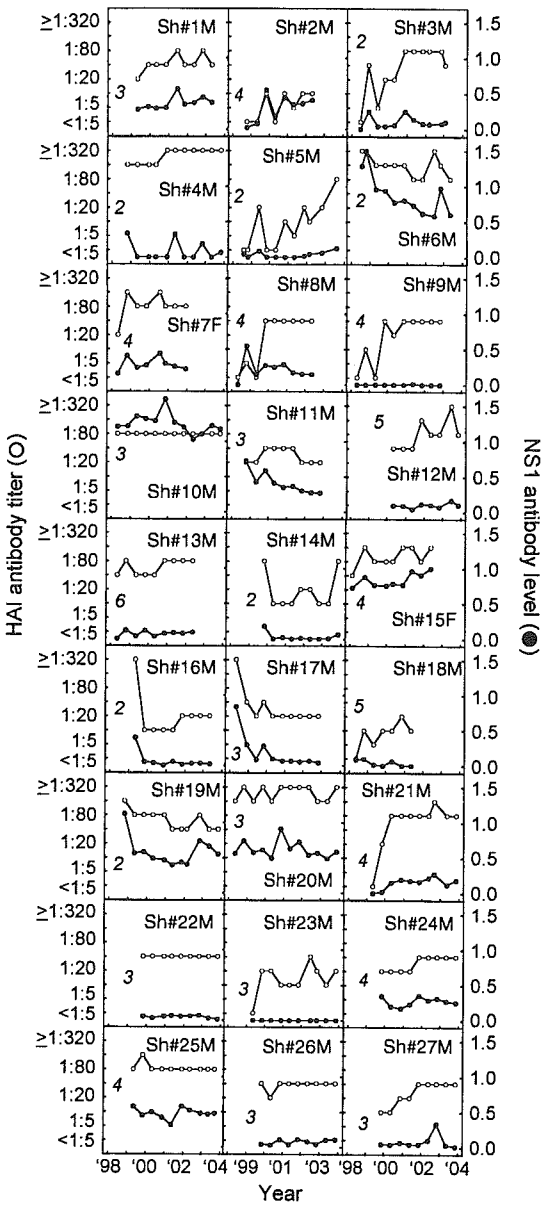

Fig. 4. Time courses of NS1 antibody (filled circle) and HAI antibody (open circle) levels in 27 individual horses in Shiga Prefecture. The label in each panel indicates the horse number and gender: for instance, the sample "Sh#1M" was collected from a male horse numbered 1 in the Shiga population. Italic numerals indicate the age (year) of horses at the start of serum collection.

differences in antibody level between the first and the second samples in these pairs were plotted in a scatter graph in relation to the NS1 antibody level of the first sample (Fig. 5). Since experimental variations of horse sera with ELISA values of approximately 1.0 were 0.3 at maximum and these variations (30% at maximum) were constantly shown for samples with ELISA values of 0.333 or more in our system [18], differences between the first and second samples in paired sera were regarded as significant when the differences were more than 30% of the first antibody levels, for samples with the first antibody levels of 0.333 or more. For samples with the first antibody level of less than 0.333, differences of 0.1 or more were regarded as significant. The significant increases in NS1 antibody levels at the half-year interval were considered to be the result of exposure to natural infections during the corresponding half-year.

Table 1 summarizes the number of paired sera with or without significant increases/decreases during epizootic (May through November) or non-epizootic (November through May) seasons. Increases in antibody level were predominantly in epizootic seasons, and decreases in non-epizootic seasons, in both Ibaraki and Shiga populations. Table 1 also summarizes mean NS1 antibody levels of the first sera and their mean differences from those of the corresponding second sera. The first antibody levels of paired sera

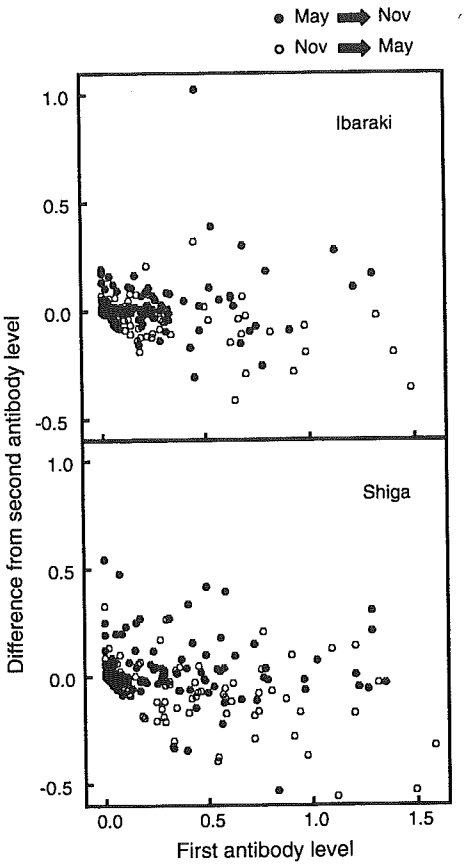

Fig. 5. The relation of NS1 antibody levels in the first samples of paired sera to their difference to those in the second samples collected a half year later. Abscissa indicates the NS1 antibody levels in the first samples and ordinate indicates the difference obtained by subtracting the antibody level of the first sample from that of the second sample. A total of 184 pairs in Ibaraki and 232 pairs in Shiga are plotted with open and filled circles for pairs whose first sera were collected in November and May, respectively.

showing increases were lower than those showing decreases. The increasing levels ranged from 0.224 to 0.264 with an average of 0.237 and the decreasing levels ranged from 0.183 to 0.315 with an average of 0.243. Since natural infections

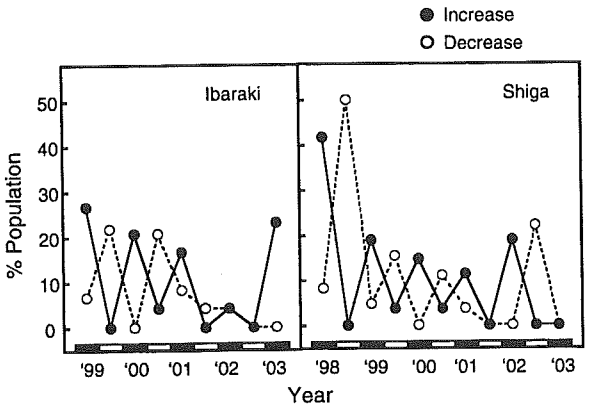

Fig. 6. Time courses of percentages of pairs that showed significant increases (filled circle) or decreases (open circle) in each of the half years from May 1998 or 1999 to November 2003. The filled bars on the abscissa indicate the period from May to November, and the open bars indicate the period from November to next May.

could occur at any time point during the half year, and antibody levels once increased may start decreasing, the levels of roughly 0.2–0.5 in our ELISA system were considered as increases in NS1 antibody level corresponding to the acquisition of natural infections in the present horse populations.

3.4. Time courses of natural infection rates in equine populations

To analyze yearly variations in natural JEV activity in Ibaraki and Shiga, infection rates in each horse population were obtained by significant increases in NS1 antibody levels during epizootic or non-epizootic seasons over 4–5 years (Fig. 6, filled circles). The time course was basically characterized by alternative changes with high and low infection rates in epizootic and non-epizootic seasons, respectively. Infection rates in epizootic seasons were mostly around 20% with an average of 18.3% in Ibaraki, and 10–20% with an average of 17.7% in Shiga. The highest rate in Shiga

Table 1
Changes in NS1 antibody levels in paired sera collected at half-year intervals

Location	Season[a]	Change	Number of pairs (%)	Mean antibody level of the first sera	Mean difference from the second sera[b]
Ibaraki	Epizootic	Increase	17 (17.0)	0.165	0.230
		Constant	79 (79.0)	0.249	0.013
		Decrease	4 (4.0)	0.423	−0.204
	Non-epizootic	Increase	1 (1.2)	0.232	0.229
		Constant	72 (85.7)	0.271	−0.024
		Decrease	11 (13.1)	0.366	−0.183
Shiga	Epizootic	Increase	20 (16.7)	0.268	0.264
		Constant	97 (80.8)	0.273	0.003
		Decrease	3 (2.5)	0.512	−0.315
	Non-epizootic	Increase	2 (1.8)	0.147	0.224
		Constant	91 (81.3)	0.292	−0.018
		Decrease	19 (17.0)	0.513	−0.270

[a] Epizootic season was defined as the period from May to November and non-epizootic season as the period from November to the next May.
[b] The difference was obtained by subtracting the antibody level of the first sample from that of the second sample.

1998 (41.7%) was significantly different from the average ($P < 0.05$), whereas the lowest rates in Ibaraki 2002 (4.2%) and Shiga 2003 (0.0%) were not so low as to result in significant differences from the average of each population ($P > 0.05$). Fig. 6 also shows percentages of pairs showing significant decreases in NS1 antibody levels (open circles); the time course was mainly opposite to that obtained with pairs showing significant increases, namely high percentages coincided with non-epizootic seasons and vice versa.

4. Discussion

The national JE surveillance program has reported a reduction in seropositivity in swine populations [9,20]. In the past (1960s) when large epidemics continued to occur [21,22], nearly 100% seroconversion was shown between late July and middle August in most of endemic areas. However, in most prefectures in recent years, seropositivity no longer reaches 100% by the end of the surveillance period (middle September). The change in the pattern of JEV activity is typically represented in Tokyo Metropolis located in east-central Japan before and after the end of the 1960s [23] and in Hyogo Prefecture located in west-central Japan before and after the early 1980s [24]. The reduction in seropositivity in swine is considered to be the result of a decrease in the number of mosquitoes and from hygienic improvements made to pig farms.

The natural JEV activity in the whole country from 1998 to 2003 [9,20] can be roughly characterized by latitude, as shown in Fig. 1: (1) mostly high levels below 35°N, (2) mostly moderate levels between 35 and 40°N, and (3) none or low levels between 40 and 45°N. This situation represents reduced JEV activity when compared with the past situation. For instance, Akita Prefecture at 40°N showed seropositivities of more than 80% in 4 years in the 1980s, whereas no seropositive swine were detected in the same prefecture in 7 of 10 years in which data were available between 1991 and 2003. Furthermore, a reduction in seropositivity in swine was also shown during the most recent 6 years in some areas below 35°N (Fig. 1).

The equine training centers in Ibaraki and Shiga are positioned at 36 and 35°N, respectively (Fig. 1). Ibaraki and Shiga both showed high swine seropositivities between 1998 and 2000 with an average of 95% (surveillance in Ibaraki was not done in 1999), whereas in 2001–2003 the mean seropositivity had decreased to 63% in Shiga and 28% in Ibaraki. The reduced virus activity is further supported by swine data from 2001 to 2003 in the Chiba and Tochigi prefectures that neighbor Ibaraki, with a mean seropositivity of 38%. Since the only prefecture (Mie) for which swine data were available, among those neighboring Shiga, still showed a high mean seropositivity of 93% in 2001–2003, the natural JEV activities in Ibaraki were more rapidly reduced than those in Shiga.

The annual change of infection rates in racehorses obtained in the present study (Fig. 6) was not always consistent with the swine data reported by the national JE surveillance program (Fig. 1). In Ibaraki, the seropositivity of swine in 2002 was higher than those in 2001 and 2003, whereas the infection rate in horses was lower in 2002 than 2001 and 2003. In Shiga, the seropositivity of swine in 2003 was high, but no infection was shown in the horses used for the present study. It seems that seropositivities among swine residing in farms far from the training center do not represent the dynamics of natural JEV activity around horses. However, the averages of natural infection rates in the present racehorse populations during epizootic seasons before 2000 and after 2001 were, respectively, 23.8% and 14.6% in Ibaraki and 25.2% and 10.2% in Shiga (Fig. 6). Thus, in terms of a tendency for natural JEV activities to reduce, swine data are consistent with the annual infection rates in horses and probably can represent a gross overview of the situation in Japan.

Induction of high levels of HAI antibodies (correlated with neutralizing antibodies) by vaccination is considered to achieve a "sterile" immunity in which any JE virus present in mosquito saliva injected in the host at the bite site is inactivated by neutralizing antibodies before infecting the host cells, and fails to produce NS1 protein. However, no significant differences were observed between individuals showing high and low HAI titers at any borderlines differentiating high from low HAI titers, in terms of the levels of increase in NS1 antibodies and the percentage of individuals showing increase in NS1 antibody level ($P > 0.05$; data not shown); suggesting that "sterile" immune responses were only induced in a small population. If sterile immunity is achieved, this increases the NS1 antibody-based infection rate.

The increases in NS1 antibody levels corresponding to acquisition of natural infection in the present horse populations are considered to be roughly 0.2–0.5 in our ELISA system. These increasing levels were much smaller than those obtained from horses in an outbreak, 1982, in Tochigi Prefecture, a neighbor of Ibaraki [18]: the mean NS1 antibody levels of four asymptomatically infected horses were 2.179 at maximum (at week 5 of serum collection) and were still high (1.395) at week 38. The reason for the difference in increasing antibody level may be the difference in the size of inoculum corresponding to the frequency of infective mosquito bites. The level obtained in the present study was equivalent to experimentally infected horses: three horses infected with 1×10^6 to 4×10^8 PFU of JEV raised antibody levels to 0.4–0.5 on days 13 and 23 [18]. Thus, the smaller increase in NS1 antibody level in the present horse population in 1998–2003 than the population in Tochigi 1982 is probably related to the reduction in the number of mosquitoes and/or the pathogenicity of wild JEV strains.

Although significant increases in NS1 antibody levels were mostly shown in epizootic seasons, a few pairs showed increases in non-epizootic seasons (Table 1, Fig. 6). An example is the pair collected from Sh#4M in November 2000 and May 2001 with antibody levels of 0.000 and 0.326,

respectively (Fig. 4). In Japan, *C. tritaeniorhynchus* has been considered as a major vector of JEV [12] in relation to the role of swine as an amplifier [3]. However, in light of the relocation and hygienic improvement of pig farms, other amplifiers and vectors might currently be involved in JEV infections of horses, and humans as well. Birds are such an effective amplifier [25]; and bats could also be a potential amplifier, since JEV has been isolated from wild bats [1,26] and experimentally infected bats developed enough viremia to infect mosquitoes [27]. A recent survey of field-collected mosquitoes carried out in Tokyo and Osaka areas reported that JEV sequences were detected in *Aedes albopictus* and *Culex pipiens* complex by polymerase chain reaction [28]. Since these mosquito species have been reported to be susceptible to oral infection with JEV [29,30] and would be active for longer periods than *C. tritaeniorhynchus* whose major habitat (rice fields) is only available for a limited period in central Japan, natural JEV infections may even occur from November to the next May, originally considered as a non-epizootic period.

Although the infection rates in the present study were obtained using significant increases in antibody level during a half year, serial sera are not generally available. Therefore, the time period from seroconversion to seroreversion is an important epidemiological factor to obtain an annual infection rate from the antibody prevalence at one time point. To estimate duration, theoretical prevalences and annual seroconversion rates were first calculated from the annual infection rate and proposed duration, and then compared with the actual data (Fig. 7). Since the calculations are made under the supposition that the prevalence and the annual infection rate are constant every year, we utilized data obtained between 1999 and 2001 when relatively constant values were shown. In 1999 to 2001, the mean prevalence was 61.9% in Ibaraki and 51.9% in Shiga. The mean annual infection rate was 20.9% in Ibaraki and 17.1% in Shiga. These infection rates were obtained by dividing the total number of pairs showing increases (14 for Ibaraki and 13 for Shiga) by the total number of pairs (134 for Ibaraki and 152 for Shiga) and then by multiplying the results by a factor of 2 (since two pairs at half-year intervals were obtained in one year for each individual). Among pairs showing increases, six (Ibaraki) and five (Shiga) pairs seroconverted, providing mean annual seroconversion rates of 9.0% in Ibaraki and 6.6% in Shiga.

Theoretical prevalences calculated from the annual infection rate and proposed duration in the Ibaraki population are shown in Fig. 7A. When the duration is 1 year, the originally seronegative population increases the prevalence to 20.9% in the first year with the value being constant after the second year; the annual seroconversion and seroreversion rates are equilibrated (20.9%). When the duration is 2 years, 20.9% of both the seropositive (20.9%) and seronegative (79.1%) populations are infected in the second year, resulting in a prevalence of 37.4% (i.e. seronegative population of 62.6%). The prevalence of 37.4% is maintained in the third year, since the percentage of individuals that acquire infection only in

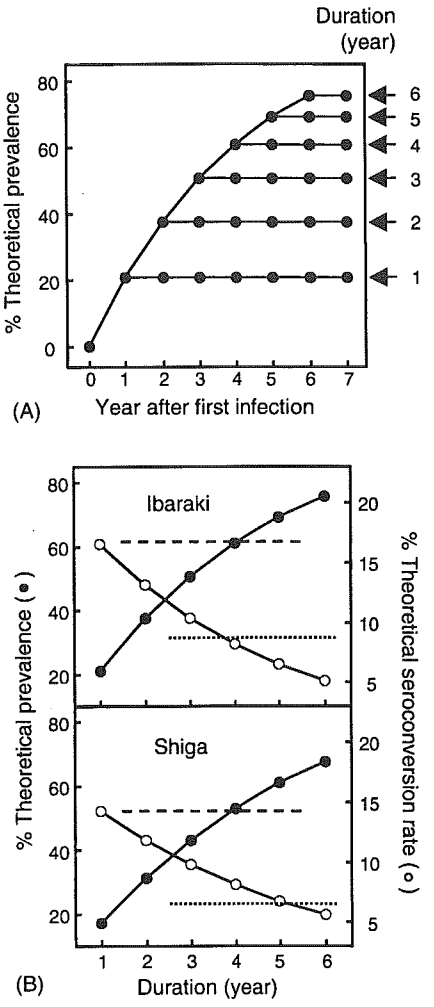

Fig. 7. Estimation of the duration of NS1 antibody responses. (A) Calculation of theoretical prevalences using the annual infection rate of the Ibaraki population (20.9%) and a proposed duration of 1–6 years. (B) Theoretical prevalences (filled circle) and seroconversion rates (open circle) in relation to the duration. The broken and dotted lines indicate levels of the actual mean prevalence and annual seroconversion rate, respectively.

the first year and serorevert in the third year (79.1% of 16.5% [the seropositive population in the second year excepting the reinfected population] which is 79.1% of 20.9% [the seropositive population in the first year]: 13.1%) is the same as the percentage of individuals that acquire infection and seroconvert only in the third year (20.9% of 62.6% [the seronegative population in the second year]: 13.1%). In a similar way, the prevalence was calculated for each of 3–6-year durations in the Ibaraki population (Fig. 7A) and so too for each of 1–6-year durations in the Shiga population (data not shown). The theoretical prevalences plotted in relation to the duration (Fig. 7B, filled circle) were compared with the actual mean prevalences in Ibaraki (61.9%) and Shiga (51.9%; Fig. 7B, broken line), estimating the duration to be approximately 4 years.

Further, theoretical seroconversion rates were calculated (Fig. 7B, open circle). In the Ibaraki population, the seroneg-

ative population is 79.1%, when the duration is 1 year, as described above. Therefore, the seroconversion rate is calculated to be 16.5% (20.9% [annual infection rate] of 79.1%). When the duration is 2 years, the seronegative population in the second year or later is 62.6% and the seroconversion rate is calculated to be 13.1% (20.9% of 62.6%). Similarly, theoretical seroconversion rates were obtained for other durations in the Ibaraki population and for all durations in the Shiga population. Compared with actual annual seroconversion rates (Fig. 7B, dotted line), the duration was estimated to be 4 and 5 years in Ibaraki and Shiga, respectively. Although estimated duration varied probably due to the small numbers of seroconverting samples in 1999–2001, the results estimated from the seroconversion rates well supported the duration (4 years) estimated from the prevalence.

The present NS1 antibody survey revealed that racehorses residing in central Japan were frequently infected with JEV, strongly suggesting that a relatively large number of mosquitoes that can transmit virus to horses and probably to humans are still present. All racehorses are vaccinated every year in Japan, and no JE cases were reported between 1986 and 2002 [11]. In 2003, one JE case appeared in a horse that was not used for racing and was not vaccinated [31]. From the results of our study, we conclude that JEV continues to actively circulate in central Japan and therefore a program of continuous vaccination is still required for protection of both horses and humans from JE.

Acknowledgements

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References

[1] Burke DS, Monath TP. Flavivirus. In: Knipe DM, Howley PM, editors. Fields virology. fourth ed. Philadelphia: Lippincott Williams & Willkins; 2001. p. 1043–125.
[2] Halstead SB, Jacobson J. Japanese encephalitis. Adv Virus Res 2003;61:103–38.
[3] Scherer WF, Moyer JT, Izumi T, Gresser I, McCown J. Ecological studies of Japanese encephalitis virus in Japan. VI. Swine infection. Am J Trop Med Hyg 1959;8:698–706.
[4] Shope RE. Medical significance of togaviruses: an overview of diseases caused by togaviruses in man and in domestic and wild vertebrate animals. In: Schlesinger RW, editor. The togaviruses. New York: Academic Press; 1980. p. 47–82.
[5] Nakamura H. Japanese encephalitis in horses in Japan. Equine Vet J 1972;4:155–6.
[6] Tsai TF, Yu YX. Japanese encephalitis vaccines. In: Plotkin SA, Mortimer Jr EA, editors. Vaccines. second ed. Philadelphia: WB Saunders; 1994. p. 671–713.
[7] Igarashi A. Japanese encephalitis: virus, infection, and control. In: Kurstak E, editor. Control of virus diseases. second ed. New York: Marcel Dekker; 1992. p. 309–42.
[8] Kitano T, Oya A. Japanese encephalitis vaccine. In: Researcher's Associates National Institute of Health, editor. Vaccine Handbook. Tokyo, Maruzen, 1996. p. 103–13.
[9] National Institute of Infectious Diseases, Department of Virology 1 and Infectious Disease Surveillance Center. Japanese encephalitis. In: Annual Report 2001, National epidemiological surveillance of vaccine preventable diseases. Tokyo, Tuberculosis and Infectious Diseases Control Division, Health Service Bureau, Ministry of Health Labour and Welfare, Government of Japan, and Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Japan, 2003. p. 75–89 [in Japanese].
[10] Hoshi S, Ito T. Statistics of equine encephalitis in Japan. Exp Rep Gov Exp Stat Anim Hyg 1951;23:1–42 [in Japanese with English summary].
[11] Livestock Industry Department, Agricultural Production Bureau. Annual Statistics of Animal Infectious Diseases. In: Statistics on Animal Hygiene 2001. Tokyo, Ministry of Agriculture, Forestry and Fisheries, 2003, p. 109 [in Japanese].
[12] Buescher EL, Scherer WF, Rosenberg MZ, Gresser I, Hardy JL, Bullock HR. Ecological studies of Japanese encephalitis virus in Japan. II. Mosquito infection. Am J Trop Med Hyg 1959;8:651–64.
[13] Fukuda H, Umehara F, Kawahigashi N, Suehara M, Osame M. Acute disseminated myelitis after Japanese B encephalitis vaccination. J Neurol Sci 1997;148:113–5.
[14] Go T, Imai T. A residual cystic lesion in acute disseminated encephalomyelitis. Neuroradiology 2000;42:682–4.
[15] Plesner AM, Arlien-Soborg P, Herning M. Neurological complications to vaccination against Japanese encephalitis. Eur J Neurol 1998;5:479–85.
[16] Konishi E, Suzuki T. Ratios of subclinical to clinical Japanese encephalitis (JE) virus infections in vaccinated populations: evaluation of an inactivated JE vaccine by comparing the ratios with those in unvaccinated populations. Vaccine 2002;21:98–107.
[17] Konishi E, Shoda M, Kondo T. Prevalence of antibody to Japanese encephalitis virus nonstructural 1 protein among racehorses in Japan: indication of natural infection and need for continuous vaccination. Vaccine 2004;22:1097–103.
[18] Konishi E, Shoda M, Ajiro N, Kondo T. Development and evaluation of an enzyme-linked immunosorbent assay for quantifying antibodies to Japanese encephalitis virus nonstructural 1 protein to detect subclinical infections in vaccinated horses. J Clin Microbiol 2004;42:5087–93.
[19] Konishi E, Pincus S, Fonseca BAL, Shope RE, Paoletti E, Mason PW. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology 1991;185:401–10.
[20] National Institute of Infectious Diseases, Infectious Disease Surveillance Center. Surveillance of swine infected by Japanese encephalitis virus in Japan, National epidemiological surveillance of vaccine preventable diseases, 2004. http://idsc.nih.go.jp/yosoku/Smenu.html.
[21] Konno J, Endo K, Agatsuma H, Ishida N. Cyclic outbreaks of Japanese encephalitis among pigs and humans. Am J Epidemiol 1966;84:292–300.
[22] Oya A. Recent tendencies of Japanese encephalitis. Pediatr Jpn 1979;20:665–9 [in Japanese].
[23] Yoshida Y, Yabuuchi K, Iwasaki K, Taguchi F. Numerical analysis for spread of the infection with Japanese encephalitis virus. J Jpn Assoc Infect Dis 1985;59:1061–72 [in Japanese with English summary].
[24] Yamaoka M, Konishi E. Recent changes in prevalence pattern of Japanese encephalitis virus in Hyogo Prefecture, Japan, using a two-dimensional distribution of IgG and IgM class antibody levels in swine sera. J Med Virol 1985;17:1–7.
[25] Buescher EL, Scherer WF, McClure HE, Moyer JT, Rosenberg MZ, Yoshii M, et al. Ecological studies of Japanese encephalitis virus in Japan. IV. Avian infection. Am J Trop Med Hyg 1959;8:678–88.
[26] Cross JH, Lien JC, Huang WC, Lien SC, Chiu SF, Kuo J, et al. Japanese encephalitis virus surveillance in Taiwan. II. Isolations from

mosquitoes and bats in Taipei area 1969–1970. J Formosan Med Asoc 1971;70:681–6.

[27] La Motte Jr LC. Japanese B encephalitis in bats during simulated hibernation. Am J Hyg 1958;67:101–8.

[28] Takasaki T, Sawabe K, Isawa H, Ito M, Kotaki T, Kurane I, et al. Detection of Japanese encephalitis virus from mosquitoes. In: Kobayashi M, editor. Studies on ecology and control measures of vectors that can transmit infectious diseases: Report 2003. Tokyo: National Institute of Infectious Diseases; 2004. p. 179–4 [in Japanese].

[29] Weng MH, Lien JC, Lin CC, Yao CW. Vector competence of *Culex pipiens molestus* (Diptera: Culicidae) from Taiwan for a sympatric strain of Japanese encephalitis virus. J Med Entomol 2000;37:780–3.

[30] Weng MH, Lien JC, Wang YM, Wu HL, Chin C. Susceptibility of three laboratory strains of *Aedes albopictus* (Diptera: Culicidae) to Japanese encephalitis virus from Taiwan. J Med Entomol 1997;34:745–7.

[31] Agricultural Production Bureau, Ministry of Agriculture, Forestry and Fisheries. Occurrence of infectious diseases in livestock. Anim Hyg Wkly 2003;55:305 [in Japanese].

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Construction of Human Fab Library and Isolation of Monoclonal Fabs with Rabies Virus-Neutralizing Ability

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Abstract: **A combinatorial human Fab library was constructed using RNAs from peripheral blood lymphocytes of 6 rabies vaccine-boosted volunteers using pComb3X phagemid vector. The size of the constructed library was approximately 7.0×10^7 *Escherichia coli* transformants. The library was selected against purified rabies virus (RV) virion or purified RV glycoprotein for isolation of phages displaying RV-neutralizing human Fab antibody. Among 132 selected clones, two Fab preparations revealed neutralizing activities against RV strain CVS when assayed in the rapid fluorescent focus inhibition test (RFFIT). The Fab preparation EP5G3 exhibited neutralizing activity with an infected cell count reduction of 76% at a dilution of 1:2, and of 20% at a dilution of 1:4. The Fab preparation GD2D12 also exhibited neutralizing activity with a 57% reduction at 1:2 and 41% reduction at 1:4. In the co-immunoprecipitation using strain CVS, the RV glycoprotein was precipitated in reactions with both Fab preparations. The RV neutralizing ability of the Fab preparations described in the study were not directly correlated with their binding specificity for RV antigens detected by ELISA.**

Key words: **Combinatorial library, Phage display, Rabies virus, Antibody**

Rabies virus (RV) is the prototype species of the genus *Lyssavirus* in the family *Rhabdoviridae*. The viral particles appear bullet-shaped under the electron microscope (12, 13). The virus consists of a surface lipid-bilayer envelope with extruded glycoprotein (G) spikes, a matrix protein (M), and an internal ribonucleoprotein (RNP). The RNP is made of a single-stranded negative-sense genomic RNA, the nucleocapsid protein (N), the virion-associated RNA polymerase (L), and polymerase cofactor phosphoprotein (P). The RNP in association with the M protein is surrounded by the envelope (30, 31).

Rabies is a zoonotic viral disease which infects wild as well as domestic animals. The virus is transmitted in mammalians through close contact with saliva from infected animals (14). The disease has been widely distributed all over the world in tropical areas. The annual number of human deaths worldwide caused by rabies is estimated to be between 40,000 and 70,000 for densely populated countries in Africa and Asia where rabies is endemic, and where successful canine rabies vaccination or control programs have not been implemented (28). According to the WHO, combined immunoglobulin-vaccine treatment is the best specific treatment available for the post-exposure prophylaxis of rabies in humans (27). Both purified equine rabies immunoglobulin and human immunoglobulin are used in rabies endemic areas; however, a comparatively high incidence of adverse reactions including serum sickness caused by heteroantigens and infectious diseases caused by undetected infectious agents are reported (27). In addition, an insufficient supply of therapeutical immunoglobulins has made post-exposure treatment more difficult.

Using the combination of a combinatorial library and phage display technology, potentially therapeutic antibodies against important viral pathogens have been iso-

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Abbreviations: CDR, complementary-determining region; CVS, challenge virus standard; FR, framework region; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; RFFIT, rapid fluorescent focus inhibition test; RNP, ribonucleoprotein; RV, rabies virus; RVG-protein, rabies virus glycoprotein; scFv, single chain Fv.

lated from humans and non-human primates (4, 7, 11, 15, 18, 20). The use of bacteriophage displaying a library of Fab or scFv antibody fragments on their surface has proved efficient for the isolation of a diverse set of human monoclonal antibodies from immune or non-immune volunteers to a variety of infectious diseases (2, 10, 22, 26). Monoclonal antibodies (MAbs) produced from such human antibody gene libraries have the potential to serve directly as immune prophylactic or therapeutic reagents against various infectious agents (2).

The current study describes the isolation of human Fab with RV-neutralizing activity from a human Fab library constructed using a peripheral blood lymphocyte pool of hyper-immune volunteers.

Materials and Methods

Virus preparation and antigen purification. RV strain Nishigahara (wild type) stocked in our laboratory was proliferated on 80% confluent BHK-21 cells grown in MEM (Invitrogen) supplemented with 3% fetal calf serum at 37 C for 4 days in T-75 flasks (Greiner). Culture fluid was clarified by centrifugation, then centrifuged at 36,000×g for 3.5 hr (SW28 rotor, Beckman). The harvested viral pellet was suspended in ca. 1.0 ml of phosphate-buffered saline (PBS, pH 7.4) and used as an antigen for panning, purifying RV glycoprotein, and immunological assays for Fab preparations. RV strain CVS (challenge virus standard, wild type) was proliferated using a standard method and used for virus-neutralizing assays and determination of RV proteins with which Fab preparations interacted in co-immunoprecipitation analysis.

RV glycoprotein purification. RV glycoprotein (RVG-protein) was purified and concentrated as described elsewhere (8). Briefly, the concentrated RV virions (ca. 2.0 mg) were dissolved in 5.0 ml of 2% octyl-β-(+)-glucopyranoside (Sigma) and the mixture was incubated at room temperature for 20 min and then centrifuged at 120,000×g for 70 min (AT4 rotor, Hitachi). The supernatant containing the extracted RVG-protein was dialyzed with a Dialysis membrane (Wako) against PBS at 4 C for 12 hr and used for panning and immunological assays for Fab preparations.

Immunization of 6 human volunteers with rabies vaccine. Six healthy volunteers, who had previously been vaccinated with rabies vaccine, were boosted two times with inactivated tissue-cultured rabies vaccine (HEP Flury strain, Kaketsuken) at the doses recommended by manufacturer at a 2-week interval. Volunteers were bled at a point pre-boosting and at 2 weeks after each boost, and neutralizing antibody titers against RV strain Nishigahara were determined by rapid fluorescent focus inhibition test (RFFIT) as described elsewhere (23). Each volunteer gave consent to participate in the study after a full explanation of the purpose and design of the study. The study was approved by the ethics committee of the Faculty of Medicine, Oita University.

Construction of human Fab antibody library. One hundred milliliters of peripheral blood was obtained from each volunteer and lymphocytes were separated on a Ficoll-paque gradient by centrifugation and pooled. Aliquots of approximately 1.7×10^7 cells were stored in liquid nitrogen. Total RNA was extracted from 3.4×10^7 lymphocytes using an RNA Extraction Kit (Stratagene) and mRNA was reverse-transcribed using oligo(dT) as a primer (ThermoScript RT-PCR System, Invitrogen).

The full-length human Fab DNA product was created by PCR as described elsewhere (1), and an overall method for constructing human Fab DNA product used in the study is depicted in Fig. 1. Briefly, the variable regions of κ light chain (V_κ) and V_H DNA fragments responsible for antigen-binding specificity were amplified from the cDNA product by PCR using a panel of 5′ sense primers and 3′ reverse primers listed in Table 1. A 30-cycle PCR at 94 C for 15 sec, 56 C for 15 sec, and 72 C for 90 sec was performed. The constant domain of κ light chain (C_κ) and Fd region (C_H1) DNA fragments was amplified from a cloned Fab template in the vector pComb3XTT, kindly provided by Dr. C.F. Barbas III, using a pair of primers (Table 1). A 20-cycle PCR at 94 C for 15 sec, 56 C for 15 sec, and 72 C for 90 sec was performed. Equimolar quantities of the V_κ and C_κ or V_H and C_H1 DNA fragments were used in the overlap assembly PCR with 15 cycles of 94 C for 15 sec, 56 C for 15 sec, and 72 C for 2 min to create the κ light chain DNA products or Fd region products. The full-length human Fab DNA products containing both κ light chain and Fd region was created in the final overlap extension PCR of 15 cycles of 94 C for 15 sec, 56 C for 15 sec, and 72 C for 2 min using the primer set listed in Table 1. The amplified full-length Fab PCR products were pooled, digested with SfiI, and cloned into pComb3X vector (provided by Dr. C. Barbas III, the Scripps Research Institute). The plasmids containing the full-length Fab PCR products were used for transformation of *Escherichia coli* XL1-Blue (Stratagene) by electroporation (E. coli pulsar, BioRad).

Panning of the phage library. Approximately 1.0×10^8 transformants of *E. coli* XL1-Blue were grown in 2YT broth (Invitrogen) containing 1% glucose, 10 μg/ml of tetracycline, and 75 μg/ml of ampicillin for 3 hr at 37 C. The bacterial culture was then infected with helper phage VCS M13 (Stratagene) at an moi of 50 to

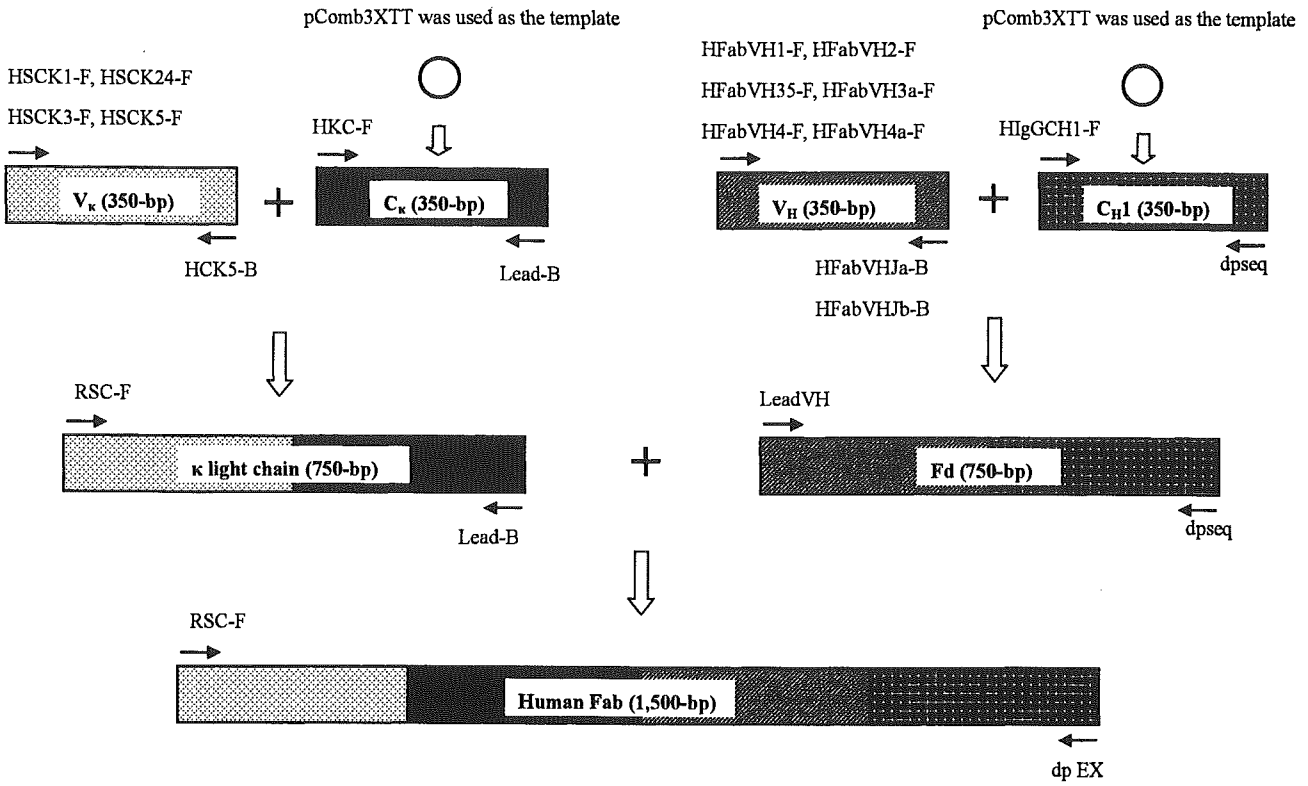

Fig. 1. PCR amplification and assembly of coding sequences of human Fab. The variable regions of κ light chain (V_κ) and Fd region (V_H) DNA were amplified from the cDNA product with the primers indicated. The constant domain of κ light chain (C_κ) and V_H heavy chain (C_H1) DNA were amplified from a cloned Fab in the vector pComb3XTT (provided by Dr. C.F. Barbas III). The amplified V_κ and C_κ products or V_H and C_H1 products were used as templates in overlap assembly PCR to create κ light chain and Fd region products, respectively. The full-length human Fab DNA product was created in the final overlap extension PCR using the constructed κ light chain and Fd region DNA products as templates (see text for details). *The figure was created by partially adapting Andris-Widhopf, J., Steinberger, P., Fuller, R., Rader, C., and Barbas III, C.F. 2001. Generation of antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences, p. 9.1–9.22. *In* Barbas III, C.F., Burton, D.R., Scott, J.K., and Silverman, G.J. (eds), Phage display: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

generate a recombinant phage library. The phage library was panned by affinity binding on RV virions coated directly on a 96-well ELISA plate (A/2 area, Corning Coaster) (ca. 16 μg protein per well) or the purified RVG-protein coated on a 35-mm tissue culture dish (Iwaki) (120 to 240 μg protein per dish), both of which were blocked with 3% nonfat powdered milk in PBS. Following five rounds of panning, the eluted phage mixture was used to infect *E. coli* XL-1 Blue and replicative form DNA (phagemid) was prepared. The nonsuppressor bacterial strain Top 10 (Invitrogen) was transformed with the selected phagemid and colonies that yielded soluble Fab were used for screening for particular clones by ELISA.

Screening for soluble Fab producing clones. The transformed Top 10 bacterial cultures of 150 μl of LB broth (Invitrogen) containing 75 μg/ml of ampicillin in a 96-well round bottom microtiter plate (Falcon) were induced with 1.0 mM isopropylthio-galactoside (IPTG) (TaKaRa) at 30 C for 5 hr, then clarified by centrifugation and used for samples for determining the soluble Fab yield by ELISA. Briefly, diluted (1:1,000 diluted) Goat anti-human IgG $F(ab')_2$ (Pierce) was coated overnight at 4 C and the centrifuged samples were added and incubated at 37 C for 1 hr. Anti human IgG (Fab specific, alkaline phosphatase-conjugated, 1:1,000 diluted) (Sigma) was used as the secondary antibody and reactions were visualized with phosphatase substrate (Sigma). A sample showing an OD reading at 405 nm more than 10-fold higher than that of the negative control (Bovine serum albumin, BSA; 500 μg/ml) was selected as a soluble Fab-producing clone. A bacterial clone producing soluble Fab was grown in 50 ml of LB-broth containing 75 μg/ml of ampicillin and 1% glucose to an early exponential phase at 30 C. The culture was induced with 1.0 mM of IPTG at 30 C for 5 hr and centrifuged, and resuspended in ca. 1.0 ml of PBS containing 1.0 mM ethylenediaminetetraacetic acid

Table 1. A list of primers used for constructing the human Fab library

Primer sets for V_H DNA fragment:
Sense:
HFabVH1-F: 5′ GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG 3′
HFabVH2-F: 5′ GCTGCCCAACCAGCCATGGCCGAGATCACCTTGAAGGAGTCTGG 3′
HFabVH35-F: 5′ GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGAGTCTGG 3′
HFabVH3a-F: 5′ GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGKTGGAGTCTG 3′
HFabVH4-F: 5′ GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG 3′
HFabVH4a-F: 5′ GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTACAGCAGTGGGG 3′
Reverse:
HFabVHJa-b: 5′ CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGGTTCC 3′
HFabVHJb-B: 5′ CGATGGGCCCTTGGTGGAGGCWGRGGAGACGGTGACCAGGGTBCC 3′
Primer sets for C_H1 DNA fragment :
Sense:
HIgGCH1-F: 5′ GCCTCCACCAAGGGCCCATCGGTC 3′
Reverse:
dpseq: 5′ AGAAGCGTAGTCCGGAACGTC 3′
Primer sets for V_κ DNA fragment:
Sense:
HSCK1-F: 5′ GGGCCCAGGCGGCCGAGCTCCAGATGACCCAGTCTCC 3′
HSCK24-F: 5′ GGGCCCAGGCGGCCGAGCTCGTGATGACYCAGTCTCC 3′
HSCK3-F: 5′ GGGCCCAGGCGGCCGAGCTCGTGWTGACRCAGTCTCC 3′
HSCK5-F: 5′ GGGCCCAGGCGGCCGAGCTCACACTCACGCAGTCTCC 3′
Reverse :
HCK5-B: 5′ GAAGACAGATGGTGCAGCCACAGT 3′
Primer set for C_κ DNA fragment :
Sense :
HKC-F: 5′ CGAACTGTGGCTGCACCATCTGTC 3′
Reverse:
Lead-B: 5′ GGCCATGGCTGGTTGGGCAGC 3′
Primer set for heavy chain Fd overlap assembly:
Sense:
LeadVH: 5′ GCTGCCCAACCAGCCATGGCC 3′
Reverse :
dpseq: see above
Primer set for light chain overlap assembly:
Sense:
RSC-F: 5′ GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC 3′
Reverse:
Lead B: see above
Primer set for PCR assembly of light chain and heavy chain (Fd) sequences:
Sense:
RSC-F: see above
Reverse:
dp-EX: 5′ GAGGAGGAGGAGGAGGAGAGAAGCGTAGTCCGGAACGTC 3′

(EDTA) and 1.0 mM phenylmethanesulfonyl fluoride (PMSF). Periplasmic Fab antibodies were released by sonication, and clarified by centrifugation at 15,300×g (MX-300, TOMY) for 30 min. The harvested supernatant was used as an Fab antibody preparation for virus-neutralizing antibody assay, ELISA for reactivity against RV virions or the purified RVG-protein, and for co-immunoprecipitation.

Virus-neutralizing assay. Serum neutralizing antibody titers of the 6 volunteers against RV strain Nishigahara were determined by RFFIT on BHK-21 cells using sera taken in the pre-boosting period or at 2 weeks after each boost. The titers were expressed as the reciprocal of the endpoint serum dilution demonstrating a 50% reduction in the number of fluorescent foci of RV-infected cells (23, 25). Each volunteer had a background of previous rabies vaccinations with distinctive vaccine products (data not shown). Neutralizing activity of Fab antibody preparations was determined by RFFIT using strain CVS on Chicken Embryo Relat-