Both were incubated at 37°C for 24 h. For inactivation of PNGase F, the samples were incubated at 65°C for 20 min.

Immunoprecipitation. The dengue viral antigens were immunoprecipitated with antibody D1-4G2 by using protein A-agarose (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The precipitated proteins were eluted in electrophoresis buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and detected by Western blotting (see below).

Western blotting. Western blotting was performed essentially as described previously (33). Briefly, samples were run on standard Laemmli gels under nonreducing conditions, transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA), and then detected by monoclonal antibodies to the E or prM protein of DENV2 or JEV.

Mouse experiment. Groups of five or six 4-week-old male ICR mice (CLEA Japan, Tokyo, Japan) were inoculated twice or three times with purified EPs or with EPs mixed with DNA plasmids by using a spring-powered needle-free jet injector (ShimaJET; Shimadzu Corp., Kyoto, Japan). The DNA plasmids used were pcDNA3, pcD2ME, and pcJEME. Mice were bled retro-orbitally, and individual sera were examined for neutralizing antibodies, unless otherwise specified. For protection experiments, immunized mice were challenged intraperitoneally with 100 50% lethal doses (LD_{50}) of the Beijing P3 strain of JEV 10 weeks after the first immunization. The challenged mice were observed for survival daily for 3 weeks. All of the animal experiments were conducted according to the Guidelines for Animal Experimentation at the Kobe University Graduate School of Medicine.

Neutralization test. Neutralizing antibody titers were determined essentially as described previously, by using plaque reduction assays performed with DENV2 strain NGC or JEV strain Nakayama in the presence of rabbit complement (19). The plaques were visualized by immunochemical staining (see above). The neutralizing antibody titers were expressed as the maximum serum dilution yielding a 70% reduction in plaque numbers.

ELISA for quantification of antibodies to DENV2 or JEV. An ELISA to quantify IgG antibodies to DENV2 or JEV was performed as described previously (31). Briefly, microplates were sensitized by incubation first with rabbit hyperimmune sera against either DENV2 or JEV and then with a DENV2 or JEV antigen. Subsequently, the microplates were incubated serially with test sera (dilution, 1:100), alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. The antigens—D2EP-Sf9, D2EP-D, JEEP-Sf9, and JEEP-F—were used at 5 ng/well. To eliminate nonspecific reactions, the absorbances obtained with nonsensitized wells were subtracted from those obtained with antigen-sensitized wells. The cutoff value, differentiating positive from negative sera, was determined by the mean plus 3 standard deviations (SDs) obtained with 14 naïve mouse sera.

Statistical analysis. Statistical analysis was performed using Student's *t* test, Fisher's exact test, or Pearson's correlation coefficient test. Probability levels (*P*) of <0.05 were considered significant.

RESULTS

***In vitro* expression of pIBD2ME and pIBJEME in Sf9 cells.** Following transfection with pIBD2ME or pIBJEME, 10 to 30% of cells were stained with the monoclonal antibodies specific for E or prM proteins (data not shown). This result indicated that pIBD2ME and pIBJEME are able to express E and prM proteins in Sf9 cells.

Biophysical/biochemical characterization of E antigens produced by Sf9 cells. Sedimentation analyses of the antigens released from pIBD2ME- or pIBJEME-transfected Sf9 cells were performed. Although these data are not shown, these analyses identified a peak corresponding to the E antigen in the Sf9 samples which coincided with that detected in pcD2ME- or pcJEME-transfected CHO cells and with the SHA particles obtained from DENV2- or JEV-infected C6/36 cells. Western blot analyses of the extracellular antigens indicated that for both DENV2 and JEV, the E protein contained in the Sf9 samples comigrated in SDS gels with the same component found in the transfected CHO or infected C6/36 samples, when these E protein samples were treated with PNGase F (data not shown). These results indicated that DENV2 and JEV antigens produced by Sf9 cells were produced in a particulate form and were equivalent to those produced by mammalian cells (CHO) and the cells of another insect (C6/36).

Immunogenicity of EPs produced by Sf9 cells. To evaluate the potential of insect cell-derived EPs to be used as vaccine antigens, these EPs were examined for their ability to induce neutralizing antibodies in mice. Two groups of six mice were immunized twice, with an interval of 6 weeks between immunizations, with 1 μg of JEEP-Sf9 either alone or mixed with 7.3 μg of pcDNA3, used as a CpG adjuvant. Three weeks after the second immunization, mice developed neutralizing antibody titers of 1:80 (without adjuvant) and 1:320 (with adjuvant), as determined from pooled sera (data not shown). Another experiment was performed with a group of six mice immunized three times at intervals of 2 weeks with a mixture of 100 ng of D2EP-Sf9 and 36.5 μg of pcDNA3. One week after the third immunization, sera pooled from these mice showed a neutralizing antibody titer of 1:20 (data not shown). Despite the limited number of animals used in these experiments, these results indicated the ability of EPs derived from insect cells to elicit neutralizing antibodies in mice.

To further evaluate the vaccine potential of insect cell-derived EPs, their ability to induce neutralizing antibodies was compared with that of mammalian-cell-derived EPs. For this evaluation, EPs were coinoculated with DNA vaccines: either pcD2ME or pcJEME. Specifically, groups of five mice were immunized twice, with a 6-week interval between immunizations, with either 100 ng of D2EP-Sf9 mixed with 50 μg of pcD2ME or 1 μg of JEEP-Sf9 mixed with 10 μg of pcJEME. In addition, the same amounts of EPs derived from CHO cells transiently or continuously expressing EPs (D2EP-CHO, D2EP-D, JEEP-CHO, JEEP-F) were used in place of insect cell-derived EPs. For reference, groups of five mice were immunized with pcD2ME or pcJEME alone. Furthermore, six mice were inoculated with phosphate-buffered saline (PBS) as a control and were used in the subsequent challenge experiment.

The time course of the mean antibody titers obtained by coimmunization of pcD2ME with D2EP-Sf9 was comparable to those obtained with D2EP-D and D2EP-CHO (Table 1). The titers at weeks 7 and 9 were approximately 8- to 16-fold higher than those obtained by immunization with pcD2ME alone, and the differences were significant (P, <0.001). Similarly, neutralizing antibody titers induced by coimmunization with pcJEME and JEEP-Sf9 were equivalent to those induced by coimmunization with pcJEME and either JEEP-F or JEEP-CHO. The mean antibody titers at week 9 were approximately 8-fold higher than that obtained by immunization with pcJEME alone. Significant differences in titers between mice immunized with DNA alone and those coimmunized with any EP preparation, except for mice coimmunized with pcJEME and JEEP-CHO, were detected at weeks 7 and 9. These results indicated that EPs produced by Sf9 cells can induce neutralizing antibodies in mice at levels similar to those induced by EPs produced by mammalian cells, when assessed in the context of coimmunization with DNA vaccines.

The protective capacity of induced neutralizing antibodies was investigated by a challenge experiment (Table 1). For this experiment, mice immunized with a mixture of JEV-EPs and

TABLE 1. Evaluation of Sf9 cell-derived EPs for immunogenicity and protective capacity in mice[a]

Expt no.	Immunogen[b]		No. of mice	Neutralizing antibody titer[c] at:				% Survival[e] (no. alive/total no.)
	EPs	DNA		Wk 3[d]	Wk 5	Wk 7	Wk 9	
1	D2EP-Sf9	pcD2ME	5	<1:10	<1:10	1:226***	1:160***	—
	D2EP-CHO	pcD2ME	5	<1:10	<1:10	1:453***	1:320***	—
	D2EP-D	pcD2ME	5	<1:10	<1:10	1:320***	1:453***	—
		pcD2ME	5	<1:10	<1:10	1:20	1:20	—
2	JEEP-Sf9	pcJEME	5	1:10	1:10	1:844***	1:844*	100 (5/5)*
	JEEP-CHO	pcJEME	5	<1:10	1:11	1:184	1:735	100 (5/5)*
	JEEP-F	pcJEME	5	<1:10	1:20	1:557**	1:1,110*	100 (5/5)*
		pcJEME	5	1:15	<1:10	1:106	1:121	—
	PBS		6	<1:10	<1:10	<1:10	<1:10	16.7 (1/6)

[a] Groups of 4-week-old male ICR mice were immunized twice, with an interval of 6 weeks between immunizations, with the indicated immunogens. As a control for the challenge experiment, mice were inoculated with PBS (Expt 2).

[b] The doses of EPs (E amounts) were 100 ng for D2EPs and 1 μg for JEEPs. The doses of DNA vaccines were 50 μg for pcD2ME and 10 μg for pcJEME.

[c] Represented as the maximum serum dilution yielding a 70% reduction in plaque number. Neutralizing antibody titers against DENV2 were obtained with pooled sera and are represented as the geometric mean titer (GMT) obtained from two separate assays (Expt 1). Neutralizing antibody titers against JEV were obtained with individual sera and are represented as the GMT (Expt 2). When GMTs were calculated, titers below the detection limit (<1:10) were assigned the value of 1:5. Asterisks indicate significant differences from antibody levels induced by DNA alone (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

[d] wk, weeks after the first immunization.

[e] Survival 3 weeks after challenge with 100 LD_{50} of the Beijing P3 strain of JEV. —, not done. Asterisks indicate significant differences from the survival rate of the PBS-inoculated group ($P < 0.05$).

pcJEME were used 10 weeks after the first immunization. All of the immunized mice were fully protected from lethal JEV challenge. Although this challenge dose resulted in one survivor in the PBS-inoculated group, the survival rates were significantly different between any vaccinated group (100%) and the PBS-inoculated group (16.7%: P, <0.05 by Fisher's exact test). These results indicated that neutralizing antibodies induced in mice by Sf9-derived EPs showed a level of protection equal to that of EPs produced by mammalian cells under our experimental conditions.

Applicability of Sf9 cell-derived EP antigens to ELISA. To evaluate the applicability of EPs produced by insect cells as antigens in immunodiagnostic tests, Sf9 cell-derived EP antigens were tested in a conventional ELISA and compared with EP antigens derived from mammalian cells (Fig. 1). For each of the antigens, D2EP-Sf9 or JEEP-Sf9, 50 mouse sera with known neutralizing antibody titers were used. Comparison of the ELISA reactivities against D2EP-Sf9 and D2EP-D provided a high correlation coefficient of 0.979 (P, <0.001). Also, a high correlation coefficient of 0.984 (P, <0.001) was obtained between ELISA values against the JEEP-Sf9 and JEEP-F antigens. These results indicated that EPs derived from Sf9 cells were antigenically equivalent to those derived from mammalian cells. Furthermore, ELISA values obtained with D2EP-Sf9 or JEEP-Sf9 antigens were significantly correlated with the titers of neutralizing antibodies against DENV2 and JEV, with correlation coefficients of 0.914 (P, <0.001) and 0.846 (P, <0.001), respectively. The consistency of the results obtained by ELISA using D2EP-Sf9 with those obtained by the neutralization test was 92.2%, with the ELISA showing 94.9% sensitivity and 88.0% specificity by comparison with the neutralization test. A similar analysis using JEEP-Sf9 antigens showed 92.2% consistency, with 93.3% sensitivity and 89.5% specificity. Although our ELISA was slightly less sensitive than the neutralization test under the present assay conditions, the significantly high correlation and consistency with this representative serodiagnostic method indicated the potential applicability of the Sf9-cell-derived EPs in immunodiagnostic tests.

Yield of EPs from transiently transfected Sf9 cells. Since one of the advantages of using insect cells is that they are able to produce recombinant proteins in larger amounts than mammalian cells, the yields of E antigen from Sf9 cells were compared with those obtained from CHO cells in a transient expression system. Following transfection with varying amounts (1 to 4 μg) of plasmids, culture supernatant samples were collected at 24, 48, and 72 h, and the amount of E antigen in the culture fluids was determined by ELISA. As shown in Fig. 2, Sf9 cells produced roughly 10-fold-higher levels of DENV2

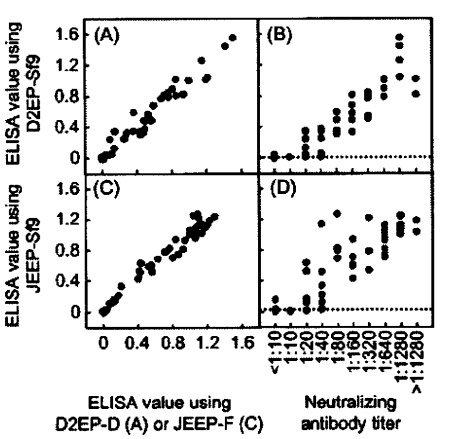

FIG. 1. Evaluation of EPs derived from Sf9 cells for use as ELISA antigens. The ELISA using Sf9 cell-derived EP antigens was compared with the ELISA using CHO cell-derived EP antigens (A and C) and with the neutralization test (B and D), using 50 mouse sera. The dotted lines indicate the cutoff levels in the ELISA to differentiate positive from negative samples: 0.008 for DENV2 and 0.014 for JEV. This cutoff level was calculated from ELISA absorbance values obtained using 14 naïve mouse sera (mean plus 3 SDs).

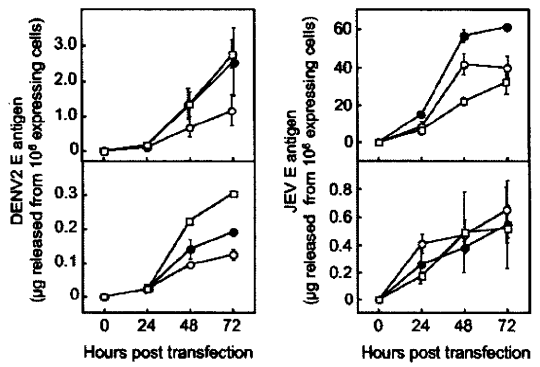

FIG. 2. Yields of E antigens from transiently transfected Sf9 and CHO cells. Sf9 cells (top) were transfected with pIBD2ME (left) or pIBJEME (right), while CHO cells (bottom) were transfected with pcD2ME (left) or pcJEME (right). Cells grown in 35-mm-diameter dishes were transfected with 1 (open circles), 2 (filled circles), or 4 (open squares) μg of each plasmid and were maintained in 2 ml of BacVector (Sf9 cells) or MEM containing 10% FBS (CHO cells). Three dishes were used for each experimental condition. At daily intervals, up to 3 days after transfection, 250-μl portions of culture fluids were sampled from two of the three dishes and were examined for E antigen levels by ELISA. The amounts of E antigen obtained from the two dishes were averaged. Cells in the other dishes were fixed and stained with antibody D1-4G2 or JE-10B4 at 24 h posttransfection in order to count the number of E-expressing cells. The ordinate indicates the amount of E antigen adjusted to that released from 10^6 E-expressing cells. The experiments were repeated twice. Each plot shows means and SDs (indicated by error bars) obtained from the two experiments.

EPs than CHO cells at 72 h posttransfection. For JEV EPs, the levels of production of EPs by Sf9 cells were markedly higher than those by CHO cells. At 72 h posttransfection, the highest yield obtained from Sf9 cells (transfected with 2 μg of pIBJEME) was approximately 100-fold higher than that from CHO cells (transfected with 1 μg of pcJEME). The expression level of intracellular E protein was approximately 25-fold higher than that of extracellular E protein in pIBD2ME-transfected Sf9 cells, whereas it was only 2-fold higher in pcD2ME-transfected CHO cells under the same conditions, at 24 h posttransfection (data not shown).

DISCUSSION

Insect cell expression systems have been well characterized for the production of recombinant proteins and have been successfully applied in life science research and the production of pharmaceutical agents (1, 6, 17, 53). The cultivation of insect cells does not require fetal bovine serum or other components of animal origin, reducing the risk of possible contamination of the vaccine product with animal pathogens, such as transmissible spongiform encephalopathy prions. Additionally, since insects are phylogenetically very distant from humans, insect-derived cells are generally considered to be less likely to be susceptible to human-pathogenic viruses than mammalian cells. Furthermore, various posttranslational modifications, such as glycosylation, can be achieved during the biosynthesis of recombinant proteins in insect cells, since the secretion pathway in insect cells resembles that of mammalian cells. Finally, it is speculated that the cytotoxicity of viral proteins against insect cells may be lower than that against mammalian cells, since DENV2 and JEV are insect-borne viruses. Thus, insect cells are considered more favorably than mammalian cells in terms of safety and high expression efficiency for the large-scale production of flavivirus vaccines and diagnostic antigens. In this study, we used Sf9 cells to evaluate the suitability of insect cells for the production of viral antigens. Since an Sf9 cell-derived human papillomavirus vaccine has already been licensed in many countries (45), Sf9 cells are a promising candidate for vaccine production.

Various subunit vaccines to prevent flavivirus diseases have been developed using several strategies. Our previous studies have demonstrated that the prM and E proteins expressed in mammalian cells using a recombinant vaccinia virus expression system (32, 33) or a continuous expression system (26, 27) were able to form EPs, which were useful both in vaccination and in serological diagnosis. Other laboratories have established high-yield expression systems for EPs of JEV or West Nile virus using the mammalian cell lines COS-1 (5, 15), RK-13 (24, 43), and CHO-K1 (52). In addition to mammalian cells, bacterial (2, 42, 54, 57), yeast (7, 8, 38), and insect (23, 36, 48, 51, 56) cells have been used to produce flavivirus vaccine candidates; most of these studies have focused on a truncated E protein or domain III of the E protein. These vaccine candidates were able to induce neutralizing antibodies and/or protection in animals. However, the E antigen produced in these expression systems appeared to be poorly immunogenic, since relatively large doses (20 to 100 μg) of the purified antigens were generally needed to induce neutralizing antibodies in mice (2, 7, 36, 38, 42, 48, 54, 57).

The baculovirus expression system is the most frequently used system for producing vaccine candidates from insect cells, including those against flaviviruses (23, 48, 51, 56). Two studies that used the baculovirus expression system to investigate EP vaccines have been reported (48, 56). Although this virus-based system achieved high-level expression and the correct formation of flavivirus E proteins, the disadvantage is that it is difficult to produce and purify antigens on a large scale, because proteases contained in the cell lysates can cause the degradation of the viral proteins of interest. In addition, contamination by a large amount of unneeded antigens and/or infectious particles derived from the vector virus is an unavoidable problem in this system targeting intracellular antigens. On the other hand, the plasmid-based EP expression system does not use infectious virus and does not lyse cells.

In this study, the immunogenicity of insect cell-derived EPs was evaluated mainly by using mice in the context of coimmunization with DNA vaccines. Coimmunization with protein (EPs) and gene (DNA) vaccines is an effective strategy for enhancing their immunogenicity. It has been demonstrated that the ability of one type of vaccine to induce neutralizing antibodies was synergistically increased by simultaneous immunization with another type of vaccine, allowing reductions in the doses of both vaccines and consequently reducing the cost (18, 19, 22). In this study, mice immunized twice with EPs obtained from transfected Sf9 cells in combination with DNA vaccines produced neutralizing antibodies, and the antibody titers were comparable to those elicited by EPs derived from mammalian cells, indicating that the immunogenicities of EPs derived from insect and mammalian cells are equivalent.

Sf9 cells produced up to 100-fold-larger amounts of JEV EPs than CHO cells under transient-expression conditions. The highest yield of JEV E antigen, approximately 60 μg, was obtained from 10^6 Sf9 cells at 72 h posttransfection. Assuming that high-density culture techniques, such as biomass support particles that allow 3×10^7 cells/cm^3, are used, 72-h cultivation using 1 liter of biomass support particles should yield 180 mg of JEV E antigen. Although we obtained only 10-fold higher levels of DENV2 E antigen in Sf9 cells than in CHO cells, high-level intracellular E antigen expression was observed. This result confirmed the ability of Sf9 cells to produce E antigen at a high level, even for DENV2, and indicated the potential for a further increase in the yield of extracellular E antigen.

In conclusion, Sf9 cells produced EPs of DENV2 and JEV that contained E antigens biochemically and antigenically equivalent to those expressed in mammalian cells. Additionally, EPs derived from Sf9 cells displayed immunogenicity and antigenicity equivalent to those of EPs derived from mammalian cells. Thus, the Sf9 cell expression system using a plasmid vector may be useful for the production of E antigens. DENV2 and JEV EPs obtained from this system are potentially safe, effective, low-cost candidates for the next generation of DNEV2 or JEV subunit vaccines.

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Needle-free jet injection of small doses of Japanese encephalitis DNA and inactivated vaccine mixture induces neutralizing antibodies in miniature pigs and protects against fetal death and mummification in pregnant sows

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ABSTRACT

Japanese encephalitis (JE) virus causes abortion and stillbirth in swine, and encephalitis in humans and horses. We have previously reported that immunogenicity of a DNA vaccine against JE was synergistically enhanced in mice by co-immunization with a commercial inactivated JE vaccine (JEVAX) under a needle-free injection system. Here, we found that this immunization strategy was also effective in miniature pigs. Because of the synergism, miniature pigs immunized twice with a mixture of 10 μg of DNA and a 1/100 dose of JEVAX developed a high neutralizing antibody titer (1:190 at 90% plaque reduction assay). Even using 1 μg of DNA, 3 of 4 pigs developed neutralizing antibodies. Following challenge, all miniature pigs with detectable neutralizing antibodies were protected against viremia. Pregnant sows inoculated with 10 or 1 μg of DNA mixed with JEVAX (1/100 dose) developed antibody titers of 1:40–1:320. Following challenge, fetal death and mummification were protected against in DNA/JEVAX-immunized sows.

1. Introduction

Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* of the family *Flaviviridae*, is a zoonotic pathogen of medical and veterinary importance [1]. It causes encephalitis in humans and horses, and abortion and stillbirth in pigs. The virus is distributed in many areas of Asia and parts of Oceania [2]. Approximately 50,000 human cases and 10,000 deaths are reported every year, mostly in countries that have no mass vaccination program [3]. In Japan, several thousand equine JE cases were reported in one year during large epizootics in the 1930s and 1940s [4]. A survey carried out in 1947 in Hamana district, central Japan, revealed that 40% of pregnant sows had stillbirths in the epidemic season [5]. Even after vaccinations started in 1948, a nationwide survey in 1969 showed that 53% of unvaccinated pregnant sows aborted as a result of JEV infection [6]. Therefore, JEV infections would cause significant economic losses to the swine industry in those pig-raising countries in Asia without vaccination programs.

JE is a vaccine-preventable disease. For humans, a formalin-inactivated purified JEV preparation produced from infected mouse brains (JEVAX) has been widely used [7]. Mass vaccination campaigns using JEVAX have successfully controlled JE epidemics in countries such as Japan, Korea and Taiwan. For veterinary use, a formalin-inactivated vaccine derived from porcine kidney cells or chick embryo cells [8], and live-attenuated vaccines derived from porcine kidney cells [9], bovine kidney cells [10], hamster kidney or lung cells [11] or quail cells [12] are used in swine or horses. In Japan, all racehorses are vaccinated every year and no JE cases have been reported since 1986 [13,14]. However, in 2003 one JE case was reported in a horse not used for racing and which was not vaccinated [15]. Also, it has been recommended that breeding sows be vaccinated and only 0–25 JEV-infected sows were reported in Japan every year during the period 1988–2007 [13,16]. The decreases in reported equine and swine JE cases following introduction of vaccines for animal use indicate the efficacies of the various programs.

The major difficulty for pig-raising Asian countries regarding the introduction of a JE vaccine program is the cost of the vaccine

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[17]. Existing JE vaccines are produced from infectious agents (JEV), thus posing safety concerns in their production. As containment facilities are required, this increases the price of the final product. Inactivated vaccines require purification and/or concentration processes, further increasing their costs. Live attenuated vaccines can be produced less expensively but have a potential risk of reversion to virulence. To address these concerns, genetically engineered vaccines have been developed [18,19].

DNA vaccines are gene-based and have advantages of durability, heat-stability, safety and inexpensiveness [20]; thus they constitute one of the most appropriate strategies for developing countries. However, only two DNA vaccines have so far been licensed for infectious diseases, and these are only for veterinary use; against West Nile virus or infectious hematopoietic necrosis virus [21]. The major drawback hampering the development of DNA vaccines is the lower immunogenicities seen in large animals and/or humans than those expected from results obtained in rodent models [22]. Relatively high DNA vaccine doses of 0.5–2.5 mg are generally used in large animals and humans when immunized by the intramuscular route [23,24]. Decreasing vaccine doses with the efficacy maintained may lead to reductions in cost. Therefore, several approaches to enhance immunogenicity of DNA vaccines have been investigated: optimization of the transcriptional elements on the plasmid backbone [25], immune plasmid adjuvant [26,27], use of new-generation delivery methods [28,29], and prime-boost strategies [20].

In our laboratory, strategies to increase immunogenicity of DNA vaccines have been investigated in flavivirus models using mice. We first found that simultaneous immunization with protein-based vaccines synergistically increased the ability of DNA vaccines to induce neutralizing antibodies in models of JEV and dengue type 2 virus [30]. Second, a needle-free jet-injection system was shown to enhance immunogenicity of DNA vaccines in a model of JEV [31]. Third, we demonstrated that needle-free injection of mixture of DNA and protein vaccines significantly increased their own immunogenicities in models of JEV [32], dengue type 1–4 viruses [33] and West Nile virus [34].

Neutralizing antibodies are the most important protective immunity against JEV [35]. Our DNA vaccines involve a strategy that coexpresses the premembrane (prM) and envelope (E) genes of JEV. Coexpression of flavivirus prM/E genes is known to induce the production of extracellular subviral particles in transfected mammalian cells that are an excellent immunogen for inducing strong neutralizing antibody responses in animals [36]. Our previous studies have demonstrated that a pcDNA3-based plasmid encoding the JEV prM/E genes (designated pcJEME) induced neutralizing antibodies and protection from lethal challenge in mice [37]. In addition, another JE DNA vaccine (designated pNJEME) based on pNGVL4a, a vector addressing field applications, had the ability to induce neutralizing antibodies also in swine [38] and monkeys [39].

The present study aimed to examine if the effect of our immunization strategy (needle-free injection of the DNA-protein vaccine mixture), already shown in mice, would also be effective in miniature pigs. Our results indicate a similar enhancing effect in these animals. Pregnant sows immunized twice with a mixture of 10 or 1 μg of the JE-DNA vaccine and a 1/100 dose of JEVAX developed neutralizing antibodies and were protected against fetal death and mummification.

2. Materials and methods

2.1. Cells

Mammalian Vero and mosquito C6/36 cells have been described [33]. Briefly, Vero cells were cultivated in a growth medium composed of Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2-95% air. C6/36 cells were grown under the same conditions as in Vero culture except for the addition of nonessential amino acids to the medium and that the cultivation temperature was 28 °C.

2.2. Viruses

The Nakayama strain of JEV [37] was used. Culture fluids harvested from C6/36 cells infected with the Nakayama strain were used for neutralization tests. The Sw/Mie/40/2004 strain [40] of JEV isolated from swine sera, which had been passaged four times in C6/36 cells, was used for protection experiments.

2.3. DNA vaccine

The pNGVL4a-based vaccine plasmid containing the *prM* and *E* genes of the JEV Nakayama strain (pNJEME) has been described [38]. The pNGVL4a vector was supplied by the National Gene Vector Laboratory (currently the Vector Core Laboratory), the University of Michigan: pNGVL4a was renamed to pUMVC4a. All DNA were purified using a Qiagen endotoxin-free DNA purification kit (EndoFree Plasmid Mega Kit; Qiagen, Hilden, Germany).

2.4. Protein vaccine

The formalin-inactivated, mouse brain-derived JE vaccine for human use (JEVAX) was purchased from Takeda Pharmaceutical (Osaka, Japan).

2.5. Animals

Miniature pigs of the Clawn strain were purchased from Japan Farm Clawn Institute (Kagoshima, Japan) and kept in the Institute for Experimental Animals, Kobe University Graduate School of Medicine (Kobe, Japan) and/or the facility for experimental animals, National Institute of Animal Health (Tsukuba, Japan). Prior to their use for experiments, the absence of hemagglutination-inhibiting (HAI) antibodies against JEV was confirmed in all animals. Four-week-old piglets (3–7 kg/pig) and 8- to 10-month-old pregnant sows (6–7 weeks after mating, 25–33 kg/pig) were used for immunization and/or protection experiments. Prior to all handlings, animals were sedated with a 1 mg/kg of azaperon (Stresnil; Daiichi-Sankyo, Tokyo, Japan) and 0.05 mg/kg of atropine sulfate (Atropine sulfate injection 0.5 mg; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), and were anesthetized with a mixture of 5 mg/kg of ketamine (Ketalar for intramuscular injection 500 mg; Daiichi-Sankyo) and a 0.05 mg/kg of xylazine (Celactal 2% injection; Bayer Medical, Tokyo, Japan). Pigs were kept in group cages at 28 ± 2 °C under a light (12 h) and dark (12 h) protocol, and fed powdered diet once a day. At the end of the experiment, pigs were pre-treated with azaperon and euthanized with an overdose intravenous (i.v.) injection of thiopental sodium (Ravonal 0.5 g for injection; Mitsubishi Tanabe Pharma Corporation) or pentobarbital sodium (Nembutal 5% injection; Dainippon Sumitomo Pharma, Osaka, Japan). All animal experiments at Kobe University were approved by the Institutional Animal Care and Use Committee (Permission number: P-060304) and conducted according to the Kobe University Animal Experimentation Regulations, while those at National Institute of Animal Health were carried out following the Guide for the Care and Use of Laboratory Animals in the National Institute of Animal Health under approval of the biosafety, animal care and ethical committees of National Institute of Animal Health (Permission number: 2006-781).

2.6. Immunization and challenge

Miniature pigs were immunized once or twice with a 3 or 7 week interval with DNA and/or protein vaccines by injecting 0.5 ml of the vaccine solution into the thigh or the base of the right ear lobe using a spring-powered needle-free jet-injector (ShimaJET attached with a 0.26 mm-diameter orifice nozzle; Shimadzu, Kyoto, Japan) or a normal syringe with a needle. The doses of pNJEME were 1–100 μg and those of JEVAX were 1/10 or 1/100 of a human dose: the inoculum size was adjusted with phosphate-buffered saline (PBS). Groups of pigs were immunized with a mixture of pNJEME and JEVAX: as controls, those inoculated with pNJEME alone, a mixture of JEVAX and pNGVL4a, pNGVL4a alone, or PBS were used. Pigs were bled from the ear vein and individual sera were examined for neutralizing and/or HAI antibodies.

Protection experiments were performed by monitoring levels of viremia of JEV after challenge with 6.3 $\log_{10}$ PFU of the JEV Sw/Mie/40/2004 strain inoculated into the base of the ear lobe by a subcutaneous route using a normal syringe with needle. Pigs were bled daily until 7 days after challenge and also on day 14 in some experiments. Plasma was isolated and examined for neutralizing antibodies and viremia levels. Pregnant sows were bled twice at pre-challenge, 2 weeks after the second immunization and post-challenge, 5 weeks after the second immunization. Pregnant sows were immunized twice with a 3-week interval, bled and challenged 2 weeks after the second immunization, and euthanized by an overdose of anesthetic agent. Fetal conditions in the uterus were assessed at 3 weeks following challenge (5 weeks after the second immunization). Presumed gestation dates on which fetuses died were calculated from their crown-to-rump lengths (CRLs) by the following formula; $X = 3 \times (Y + 21)$, where X is the presumed period (day) between pregnancy and the death and Y is the CRL (cm) obtained from the dead fetus.

2.7. Serological tests

Neutralizing antibodies in heat-inactivated sera/plasma were titrated using plaque reduction assays performed with JEV (Nakayama) without complement as previously described [37]. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 90% reduction in plaque number. The hemagglutination-inhibition tests were performed as previously described [31]. For treatment with 2-mercaptoethanol (2-ME), a 0.4 ml aliquot of serum was mixed with an equal volume of 0.2 M 2-ME in 0.01 M PBS. After 1 h incubation at 37 °C, the sera were extracted twice with acetone, dried in vacuo for 5 min and then restored in 0.4 ml of PBS [41]. Some sera were subjected to sucrose density gradient centrifugation to obtain IgG and IgM fractions as described previously [31]. Briefly, sera were applied to a 10–40% continuous sucrose gradient. Following centrifugation, fractions were collected from the bottom. Each fraction was tested for IgG and IgM class antibody levels by a conventional enzyme-linked immunosorbent assay using peroxidase-conjugated anti-swine IgG (whole IgG-reactive; MP Biomedicals, Irvine, US). A "synergistic" effect was defined as a "more than additive" effect: this was used when the mean neutralizing antibody titer obtained from the group of interest was significantly higher than the sum of those obtained from another two groups.

2.8. Statistical analysis

Significance of differences in geometric mean antibody titers or infective titers were evaluated by Student's t-test. For this evaluation, titers less than the detection limit (<1:10) were assigned a value of 1:5. The chi-square test with the Yates' correction factor was used to compare the percentages of abnormal fetuses between vaccinated and unvaccinated sows. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. Preliminary experiments

Since the DNA/protein vaccine mixture had been injected into the thigh in our mouse experiments, this site was selected for the first preliminary experiment using miniature pigs. Two 4-week-old piglets were immunized twice at a 7-week interval: one with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX and another with a mixture of 1 μg of pNJEME and a 1/100 dose of JEVAX. As shown in Fig. 1A, the piglet immunized with a higher dose induced a neutralizing antibody titer of 1:20 at 2 weeks after the first immunization, which was increased to 1:640 at 3 weeks after the second immunization. In addition, even 1 μg of pNJEME induced a neutralizing antibody titer of 1:160 at 2 weeks after the second immunization.

In our second preliminary experiment, the base of the ear lobe was used as an injection site for vaccination. Each of three piglets received either a single immunization with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX, or a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX (Fig. 1B). The former two piglets induced neutralizing antibody titers of 1:10–1:40, while the latter did not induce detectable levels until the end of this experiment. Based on these results, the base of the ear lobe was used as an injection site in subsequent experiments.

To confirm the reproducibility of the above experiments, a third preliminary experiment was performed using another three piglets and exactly the same immunization protocol as used in the above experiment (Fig. 1B). As shown in Fig. 1C, all piglets developed higher neutralizing antibody titers than those shown in Fig. 1B, probably due to individual variations. To analyze these quick and high neutralizing antibody responses, sera collected at 1 and 5 week were examined for IgM and IgG class antibodies separated on a sucrose density gradient. While the neutralizing antibody titers of the IgM fractions were higher than those of the IgG fractions at 1 week after immunization, the IgG fractions showed higher titers than the IgM fractions did at 5 weeks after immunization (Fig. 1D). The presence of IgM antibodies in the 1-week samples was confirmed by 8-fold decrease of HAI antibody titers after treatment of the sera with 2-ME (Fig. 1E). These results suggested that despite the considerable individual variations the needle-free immunization with the mixture of DNA/protein vaccines could induce neutralizing antibodies in miniature pigs.

3.2. Effect of needle-free injection and co-immunization with JEVAX on immunogenicity of DNA vaccine

To evaluate the effect of needle-free injection and co-immunization with JEVAX on immunogenicity of pNJEME in miniature pigs, six groups of two 4-week-old piglets were immunized twice with a 3-week interval with DNA and/or protein vaccines using a needle-free injector or a normal syringe with a needle (Fig. 2A). The DNA was 10 μg of pNJEME, while the protein was a 1/100 dose of JEVAX with or without 7 μg of pNGVL4a. pNGVL4a was added to adjust the adjuvant effect of the CpG motif contained in this vector plasmid on the immunogenicity of JEVAX. The molarity of CpG motifs contained in 10 μg of pNJEME corresponded to that contained in 7 μg of pNGVL4a.

Fig. 2B shows the time courses of the neutralizing antibody titers induced in individual piglets until 8 weeks after the first immunization. Co-immunization with the pNJEME-JEVAX mixture using a normal syringe with a needle (group 3) induced neutralizing antibodies after the second immunization in both piglets with geo-

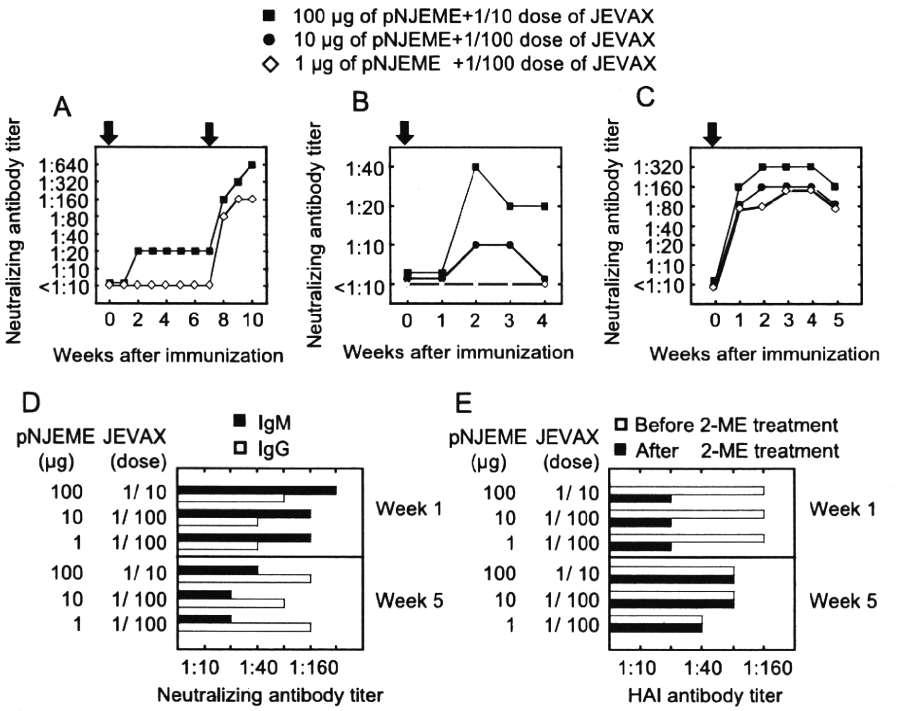

Fig. 1. Preliminary evaluation of the needle-free immunization protocol. (A) to (C) Four-week-old miniature pigs were immunized with a mixture of 100 μg of pNJEME and a 1/10 dose of JEVAX (closed squares), 10 μg of pNJEME and a 1/100 dose of JEVAX (closed circles) or 1 μg of pNJEME and a 1/100 dose of JEVAX (open diamonds). Arrows indicate the timing of immunization. The vaccine mixture was inoculated using a needle-free injector at the thigh (A) or the base of the ear lobe (B, C). (D) Sera obtained from piglets shown in Fig. 1C at week 1 and 5 were separated into IgG (open bars) and IgM (closed bars) fractions on a sucrose density gradient, and examined for neutralizing antibodies. (E) Sera used in Fig. 1D were examined for HAI antibodies with (closed bars) or without (open bars) 2-ME treatment.

metric mean neutralizing antibody titers of 1:14–1:20, while those immunized with the same dose but by using a needle-free injector (group 4) showed significantly higher mean titers of 1:95–1:190 ($P < 0.01$ by the Student's t-test at any of the 1–5 week periods after the second immunization), indicating the effectiveness of the needle-free injection method. In addition, since piglets immunized with DNA or protein vaccine alone (groups 1, 2 and 5) did not develop detectable levels of neutralizing antibodies at most time points, the titers shown by co-immunization with the pNJEME-JEVAX mixture using a needle-free injector (1:95–1:190) indicated a synergistic increase in neutralizing antibody responses, after the second immunization ($P < 0.01$ by the Student's t-test, comparing the titers obtained in group 4 with the sum of those obtained in groups 2 and 5). These results were consistent with the HAI antibody titers obtained with the same samples (Fig. 2C). Thus, the highest neutralizing antibody responses in group 4 indicated a combined effect of the needle-free injection and co-immunization with JEVAX on immunogenicity of pNJEME in miniature pigs.

During or at the end of the immunization experiment, one piglet immunized with JEVAX alone (group 5) and two piglets inoculated with pNGVL4a (group 6) suffered accidental deaths that were unrelated to the experiments. Although the piglets in group 6 were supposed to serve as a control for the challenge experiment, we did challenge the rest of the piglets at 5 weeks after the second immunization to compare viremia levels among animals immunized with different immunogens by different injection methods. Following challenge, all three piglets immunized with pNJEME by a normal syringe/needle injection (Fig. 2D, group 1) or JEVAX by a needle-free injection (group 5) showed viremia between 2 and 4 days after challenge at the maximum infective titer of 3.4 $\log_{10}$ PFU/ml. None of these piglets showed detectable levels of neutralizing antibodies prior to challenge. On the other hand, all piglets immunized with the pNJEME-JEVAX mixture, and showing individual neutralizing antibody titers of 1:10–1:320, were fully protected from viremia using any injection system (groups 3 and 4). Piglets immunized with pNJEME alone by a needle-free injector (group 2) showed lower viremia levels than those immunized with the same immunogen by a normal syringe/needle injection: one showed only 1.0 $\log_{10}$ PFU/ml at maximum and another did not develop detectable levels of viremia. These results indicated that immunization with the pNJEME-JEVAX mixture at the present dose provided miniature pigs full protection from viremia.

Fig. 2E shows time courses of neutralizing antibody titers following challenge. Piglets immunized with the pNJEME-JEVAX mixture by a needle-free injector (group 4) started increasing their antibody titers on day 3 or 4 postchallenge, reaching ≥1:1280 on day 5 or 6. By contrast, the increases in antibody titers in piglets immunized with either pNJEME or JEVAX (groups 1, 2 and 5) started on day 5 or 6 in most cases and the titers shown on day 7 were lower than those shown in group 4 piglets. Piglets immunized with the pNJEME-JEVAX mixture by a normal syringe/needle injection (group 3) showed an intermediate pattern: the start of increase in antibody titers was seen on day 4 or 5. These results indicated that miniature pigs immunized with the pNJEME-JEVAX mixture by a needle-free injector displayed strong anamnestic neutralizing antibody responses to the challenge.

3.3. *Viremia protection by 1 μg of pNJEME mixed with JEVAX*

To examine if a lower dose of the DNA vaccine would induce viremia protection, 1 μg was used as the dose of pNJEME. In this experiment, only the DNA and protein mixture was used as an immunogen, and was inoculated by using only a needle-free injector. Specifically, three groups of 4-week-old piglets were

A

Group	pNJEME	JEVAX	pNGVL4a	Needle
1	10 μg	-	-	+
2	10 μg	-	-	-
3	10 μg	1/100 dose	-	+
4	10 μg	1/100 dose	-	-
5	-	1/100 dose	7 μg	-
6	-	-	7 μg	-

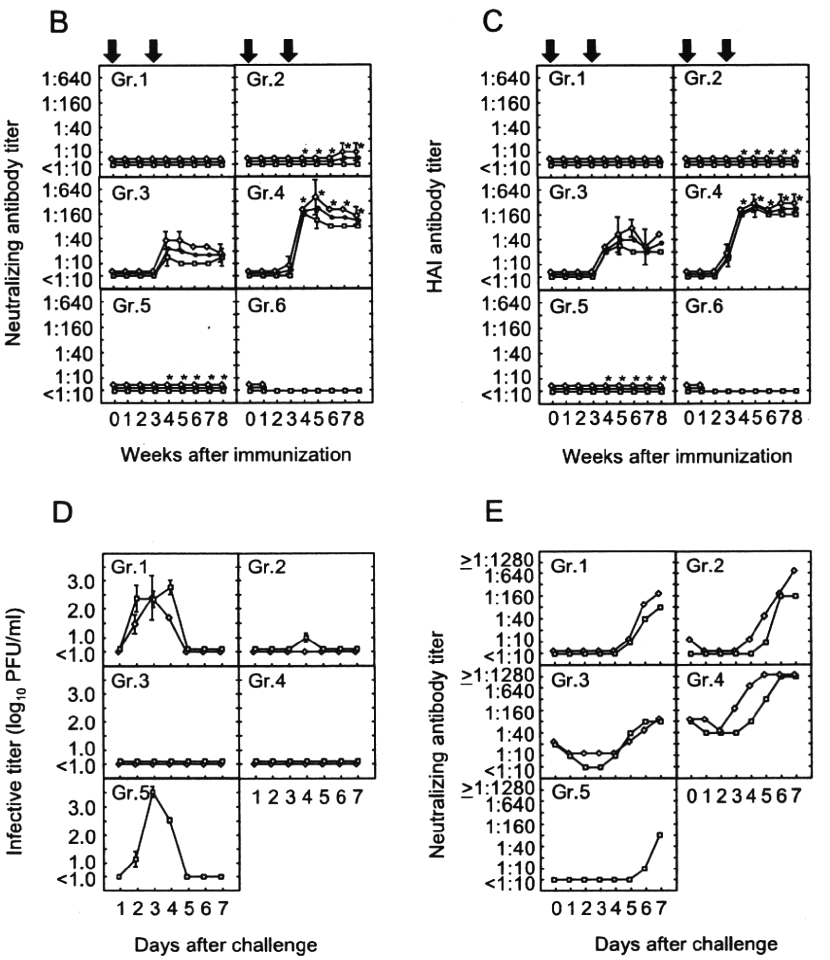

Fig. 2. Effect of the needle-free co-immunization strategy on immunogenicity and protective capacity of pNJEME in miniature pigs. Six groups of two 4-week-old piglets were immunized twice with a 3-week interval at the base of the ear lobe. Five weeks after the second immunization, piglets were challenged with 6.3 $\log_{10}$ PFU of JEV. (A) Immunogens and the injection methods used in each group. In the "Needle" column, "+" and "−" indicate the use of a normal syringe/needle and needle-free injector, respectively. (B) Time course of neutralizing antibody titers. (C) Time course of HAI antibody titers. Arrows indicate the timing of immunization. (D) Plasma infective titers following challenge. (E) Postchallenge neutralizing antibody titers. Constant symbols (open diamonds or open squares) were used in panels B-E to represent data obtained from the same individuals in each group. In panels B-D, individual data are indicated by the mean and standard error (indicated by bars) obtained from two repeated tests. Closed circles indicate the mean antibody titers obtained from two piglets in each group. The asterisks in panel B and C indicate the significance of differences detected by the Student's *t*-test ($P < 0.01$) between the mean titers shown by piglets of group 4 and the sum total of those shown by piglets of groups 2 and 5 at each time point.

immunized twice with a 3-week interval with 10 ($n = 3$) or 1 μg ($n = 4$) of pNJEME mixed with a 1/100 dose of JEVAX, as well as PBS as a control ($n = 3$). These piglets were challenged at 2 weeks after the second immunization and examined for viremia (Fig. 3). Prior to the challenge, all three piglets immunized with 10 μg of pNJEME or three of the four piglets immunized with 1 μg of pNJEME had detectable levels of neutralizing antibodies (Fig. 3, upper panels, day 0).

Piglets inoculated with PBS showed viremia titers of 2.1–5.0 $\log_{10}$ PFU/ml during days 1–4 postchallenge (Fig. 3, lower panel). All three piglets immunized with 10 μg of pNJEME were protected from viremia: the full protection induced by 10 μg of pNJEME was consistent with the results in Fig. 2D. The 1 μg of pNJEME protected viremia in three of the four piglets: the three possessed detectable prechallenge neutralizing antibodies. These results indicated that the presence of detectable levels of neutralizing antibodies in prechallenge sera correlated with protection against viremia in miniature pigs following challenge.

Neutralizing antibody titers started increasing on days 2–4 postchallenge in piglets immunized with 10 μg of pNJEME, while

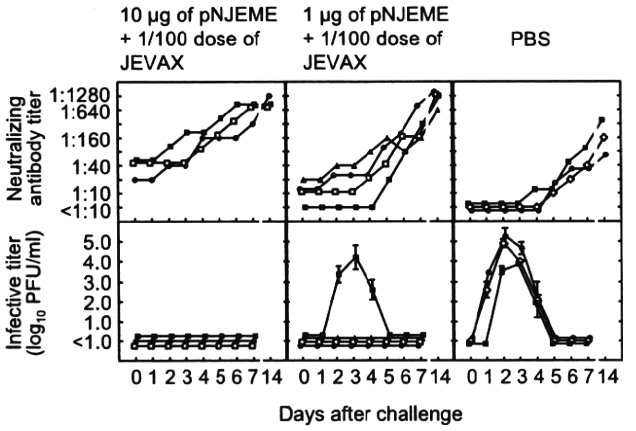

Fig. 3. Protective capacity of 1 μg of pNJEME against viremia in miniature pigs applying a needle-free co-immunization strategy. Three groups of 4-week-old piglets were immunized twice with a 3-week interval at the base of the ear lobe. Immunogens were a mixture of 10 μg of pNJEME and a 1/100 dose of JEVAX (left panels: $n=3$), 1 μg of pNJEME and a 1/100 dose of JEVAX (middle panels: $n=4$) or PBS as a control (right panels: $n=3$). Two weeks after the second immunization, pigs were challenged with 6.3 $\log_{10}$ PFU of JEV. Constant symbols were used for representing data obtained from the same individuals in each group. For infective titers, each datum indicates the mean and the standard deviation (indicated by bars) obtained from two repeated tests.

the increase in piglets inoculated with PBS started on days 4–5; immunized piglets displayed anamnestic antibody responses (Fig. 3, upper panels). Three piglets immunized with 1 μg of pNJEME that had prechallenge antibodies and were protected from viremia showed increases in antibody levels starting from days 2–4. By contrast, the start of the increase in antibody titers in the other piglet, which was immunized with 1 μg of pNJEME but did not develop detectable prechallenge antibodies and was not protected from viremia, was on day 5.

Collectively, in most cases no viremia was detected in sera showing neutralizing antibody titers of 1:10 or higher during the course of infection. Viremia disappeared when neutralizing antibody titers started increasing. Induction of anamnestic neutralizing antibody responses correlated with the prechallenge neutralizing antibody titers. These results indicated that the dose of 1 μg of pNJEME is effective for inducing neutralizing antibodies and protection against viremia in three of the four 4-week-old miniature pigs, when the DNA was mixed with a 1/100 dose of JEVAX.

3.4. Protection of pregnant sows from fetal death following challenge with JEV

To evaluate the ability of the pNJEME-JEVAX mixture to prevent miniature pigs from JEV-induced abortion, vaccinated or unvaccinated pregnant sows were challenged. In this experiment, three groups of two 8- to 10-month-old pregnant sows (6–7 weeks after mating) were immunized twice with a 3-week interval, and challenged 2 weeks after the second immunization. Immunogens were 10 or 1 μg of pNJEME mixed with a 1/100 dose of JEVAX, or PBS as a control, and were inoculated using a needle-free injector. Pregnant sows were bled twice with a 3-week interval before and after challenge (2 and 5 weeks after the second immunization), respectively. Immediately after the second bleeding, sows were euthanized and fetus conditions in the uterus were observed (Table 1).

Pregnant sows immunized with a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX developed neutralizing antibody titers of ≥1:40 at 2 weeks after the second immunization. On the other hand, inoculation with PBS did not induce detectable levels of antibodies. Following challenge, none of the twelve fetuses in immunized animals were dead or abnormal, whereas six (50%) of twelve fetuses in unimmunized sows were mummified: statistical significance ($P<0.05$) was detected in the population of dead fetuses between immunized (0%: 0 of 12 fetuses) and unimmunized (50%: 6 of 12 fetuses) groups. Although the average fetus number in a sow in the vaccinated group (3 fetuses) was different from that of the unvaccinated group (6 fetuses), both numbers were within the individual variations occurring in the Clawn strain of miniature pigs: the mean and standard error are reported as 4.7 and 2.2, respectively [42]. These results indicated that two immunizations with a mixture of 10 or 1 μg of pNJEME and a 1/100 dose of JEVAX induced neutralizing antibodies in pregnant sows and protected their fetuses from fetal death.

Attempts to demonstrate the virus or viral RNA in the brain of mummified fetuses failed, probably because of the necrosis of this tissue at the time of euthanasia (3 weeks following challenge). Since miniature pigs of the specific pathogen-free grade were used for the experiment in an appropriate animal facility, it is highly probable that JEV infection was the cause of the fetal deaths. To support this, a further attempt was done to assess the date on which fetuses died, by measuring the CRL, which is generally used for estimating the age of pig fetuses during the various developmental stages [43]. Based on the CRL, it was suggested that the mummified fetuses had died between 3 and 7 days after challenge (Table 1). The presumed death dates were within a limited period (within 5 days) and also a few days after the viremic period that was shown in the same strain of this animal (Figs. 2 and 3). These results suggested that these fetuses died of infection following challenge of the pregnant sows.

4. Discussion

Swine are the most important amplifiers of JEV in the peridomestic environment: they develop levels of viremia high enough to supply the virus to vector mosquitoes. Humans are infected

Table 1
Protective capacity of the pNJEME-JEVAX mixture against fetal death in pregnant sows.

Group	Immunogen		Pig Number	Neutralizing antibody titer		HAI antibody titer		Fetus status 3 weeks after challenge			
	pNJEME (μg)	JEVAX (dose)		2 weeks[a]	5 weeks[a]	2 weeks[a]	5 weeks[a]	Mummy	Normal	% Mummies	Presumed death date[b]
1	10	1/100	AN35	1:57	≥1:1280	1:40	≥1:1280	0	3	0	
			AO28	1:320	>1:1280	1:320	≥1:1280	0	3	0	
2	1	1/100	AO51	1:80	1:226	1:57	1:226	0	2	0	
			AO64	1:40	1:905	1:40	1:905	0	4	0	
Total								0	12	0[c]	
3	PBS		AN42	<1:10	1:80	<1:10	1:160	4	3	57	3, 4, 6, 7
			AP41	<1:10	1:40	<1:10	1:40	2	3	40	3, 6
Total								6	6	50[c]	

[a] Weeks after the second immunization. "2 weeks" indicates the timing immediately before JEV challenge. "5 weeks" indicates the time of euthanasia.
[b] Presumed death dates were estimated individually from the CRL of the mummified fetus.
[c] $P<0.05$.

by infective mosquito bites and can develop clinical symptoms at subclinical: clinical infection rates of 1:25 to 1:1,000 [44]. Therefore, mass immunization of swine is theoretically a logical strategy to reduce the viral transmission cycle in peridomestic areas, thus potentially contributing to public health and the domestic economy. Although birds or other potential amplifiers such as wild boars [45] or bats [46] may also increase the population of infected mosquitoes, a field trial conducted in an isolated small island in Japan suggested the possible effect of swine immunization on the prevention of human JEV infections [47].

In addition to its important role in virus transmission, JEV viremia has a role in virus dissemination in the host body. In humans and horses, JEV replicated at or near the mosquito bite site is transported via the blood stream to the brain where the virus may penetrate the blood-brain barrier and cause encephalitis. Similarly, intrauterine infection in swine is considered to result from virus dissemination through viremia. Therefore, the ability of neutralizing antibodies to protect against abortion/stillbirth in pigs is attributed to the reduction in viremia to levels that will not encourage virus dissemination to the uterus: similar to their ability to protect against encephalitis in humans and horses. Vaccination that can induce neutralizing antibodies is an effective measure to suppress viremia and protect humans and animals from contracting the disease. In the present study, all pigs showing a neutralizing antibody titer of 1:10 or higher were protected from viremia following challenge, consistent with a titer (1:10) known to be required for disease protection in vaccinated humans [48].

Miniature pigs have been developed as useful laboratory animals for their size. To the best of our knowledge, this is the first study using miniature pigs as an animal model to evaluate DNA vaccines for flaviviruses. In this study, pregnant sows (Table 1; approximately 9 months old) induced higher neutralizing antibody titers than did younger pigs (Fig. 2B; approximately 2 months old): immune systems of swine are known to develop as they age [49]. Thus, swine of reproductive ages may effectively induce neutralizing antibodies even by immunization with 1 μg of DNA (with a small amount of protein); however, the present study showed the ability of this low dose (1 μg) to induce neutralizing antibodies in just two pregnant sows. Optimization of the timing for immunization would be an important issue as a next step.

Needle-free injections have been used to improve the immunogenicity of DNA vaccines [29]. They include particle bombardment by a gene gun [50,51], electroporation [52,53], and high-pressure jet-injection delivery [54,55]. Both gene gun and electroporation devices have advantages of providing DNA vaccines with high immunogenicity and transfection efficiency; thus, only a small amount of DNA is required. With a gene gun, several studies have demonstrated that 1–10 μg of DNA vaccines induced neutralizing antibody titers against hepatitis B virus [56] and influenza virus [57]. However, gene guns require an expensive apparatus with gold particles and a special source of compressed helium gas, and thus are considered difficult to introduce into developing countries. As for electroporation, potential genome integration presents serious safety concerns [58]. On the other hand, spring-powered jet-injection devices are convenient and inexpensive and do not require special apparatus. Also, several studies have proven that the use of a jet-injection delivery system increased immune responses in large animals [59,60] and humans [55].

In conclusion, the present study verified the effectiveness in miniature pigs of needle-free jet injection of a JE DNA/protein vaccine mixture. While our earlier study, which did not use a needle-free injector, used 100–450 μg of pNJEME for inducing neutralizing antibodies in swine [38], the present study showed that only 10 or 1 μg of pNJEME induced neutralizing antibodies when mixed with a small amount of JEVAX and inoculated using a needle-free injector. The protective effect of the induced neutralizing antibodies against fetal death was demonstrated using pregnant sows. Field trials using a larger number of pigs will progress the evaluation of the effectiveness of the present vaccination strategy for preventing abortion and stillbirth in swine, and potentially for reducing human JEV infections.

Conflict of Interest statement

The needle-free injector, ShimaJET, used in this study is a product of Shimadzu Corporation that the authors H. Udagawa and Y. Mukuta are employees of. However, sale of the apparatus has been halted since 2007. The injector was remodeled, in part for this study, with support from the above noted JST grant. The other authors declare they have no conflicts of interest.

Acknowledgments

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Protocols

Non-structural protein 1 (NS1) antibody-based assays to differentiate West Nile (WN) virus from Japanese encephalitis virus infections in horses: Effects of WN virus NS1 antibodies induced by inactivated WN vaccine

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ABSTRACT

Antibodies to non-structural protein 1 (NS1) of West Nile virus (WNV) have been used to differentiate WNV infection from infection by serologically cross-reactive flaviviruses, including Japanese encephalitis virus (JEV), in horses. However, since the inactivated West Nile (WN) vaccine has been reported to induce NS1 antibodies, there is concern about the reliability of using NS1-based assays for testing vaccinated horses. Therefore, the effect of inactivated WN vaccine-induced antibodies on an epitope-blocking ELISA and complement-dependent cytotoxicity (CDC) assay were investigated. Both assays are based on NS1 antibodies and were established previously to differentiate WNV from JEV infections in horses. Groups of three horses were vaccinated with two or three doses of a commercial inactivated WN vaccine and NS1 antibodies were detected by a conventional ELISA after the second vaccination. Vaccine-induced NS1 antibodies were also detected by blocking ELISA and a CDC assay and affected the ability of these assays to differentiate WNV from JEV infections. However, the effect was less significant in the CDC assay, where use of a low serum concentration ensured effective differentiation. The more efficient detection of infection-induced antibodies over vaccine-induced antibodies by the CDC assay was potentially attributable to the different IgG isotype profiles of these antibodies.

1. Introduction

West Nile (WN) fever and WN encephalitis are serious diseases in humans and horses. The causative agent is WN virus (WNV), a member of the *Flavivirus* genus, which belongs to the *Flaviviridae* family. WNV also belongs to the Japanese encephalitis (JE) serocomplex, which includes Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV) and Saint Louise encephalitis virus (SLEV) (Gubler et al., 2007). The rapid expansion of WNV in the Western hemisphere, following the invasion of this virus into the USA in 1999 (Petersen and Hayes, 2008), prompted the Japanese government to create manuals and guidelines for serological testing of WNV (Kobayashi and Kurane, 2003; Kurane, 2005; Ministry of Agriculture, Forestry and Fisheries and Ministry of Health, Labour and Welfare, Japan, 2003). An inactivated vaccine was approved in preparation for the arrival of WNV in Japan.

JEV is currently an infectious cause of disease in Japan. Since disease manifestations are similar between WNV and JEV infections (Castillo-Olivares and Wood, 2004; Endy and Nisalak, 2002; Gould and Solomon, 2008), laboratory tests are essential for differential diagnosis of WNV from JEV infections. Although virus isolation or viral RNA detection methods produce a firm diagnosis, the short time period of viremia or RNAemia limits the use of these methods (Sejvar and Marfin, 2006). Thus, antibody testing constitutes a more practicable alternative. However, diagnosis of WNV infection by detection of antibodies is complicated by the serological cross-reactivity among the JE serocomplex, even when using a neutralization test that provides high specificity (Kuno, 2003). For instance, when individuals preimmune to JEV were infected with WNV in animal models, including mice (Lim et al., 2008), horses (Shirafuji et al., 2009) and pigs (Williams et al., 2001), high levels of cross-reactive neutralizing antibodies to JEV were induced by anamnestic responses, which were equivalent to, or even higher than, antibody levels induced against WNV.

Antibody assays targeting the non-structural protein 1 (NS1) have been established to overcome the diagnostic problems caused by the cross-reactivity of flaviviruses (Blitvich et al., 2003; Hall et al., 1995; Kitai et al., 2007, 2010). Flavivirus NS1 contains more

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species-specific antigenic epitopes than the envelope (E) protein (Hall et al., 1990). In fact, western blot analyses using WNV-positive human plasma and chicken sera and dengue virus-positive human sera indicated that NS1 was more species-specific than the E protein (Oceguera et al., 2007). Using an NS1-based strategy, epitope-blocking ELISAs have been established to differentiate WNV infections from infections with SLEV (Blitvich et al., 2003) or MVEV (Hall et al., 1995). More recently, an epitope-blocking ELISA (Kitai et al., 2007) and a complement-dependent cytotoxicity (CDC) assay (Kitai et al., 2010) have been developed using this strategy to differentiate WNV from JEV infections in horses. The ability of these two assays to differentiate WNV from JEV infections has been demonstrated based on the assay cutoff that was obtained by using WNV antibody-negative control horses including those naturally infected with JEV. However, a drawback of the NS1-based strategy is that assays may be greatly affected by a vaccine that induces NS1 antibodies in the host. In horses, it was reported that the inactivated WN vaccine (Innovator, Fort Dodge Animal Health) induced antibodies to NS1 but a recombinant canarypox WN vaccine (Recombitek, Merial Limited) did not (Balasuriya et al., 2006). The present study aimed to investigate the effects of NS1 antibodies induced by the inactivated WN vaccine on the ability of the blocking ELISA and CDC assay to differentiate WNV from JEV infections.

2. Materials and methods

2.1. Virus strain

The NY99 strain of WNV, isolated from an infected horse, was obtained from the National Veterinary Services Laboratories, United States Department of Agriculture (Ames, IA, USA). This strain was passaged twice through Vero cells (JCRB9013: Japanese Collection of Research Bioresources, Osaka, Japan) and the infected culture fluids were used for neutralization tests.

2.2. Monoclonal antibodies

A monoclonal antibody, WN-2H4, specific for NS1 of WNV (Kitai et al., 2007) and monoclonal antibodies specific for equine IgGa, IgGb, IgGc and IgG(T) (Sugiura et al., 1998) have been described previously. These monoclonal antibodies were obtained in an ascites form from pristane-primed BALB/c mice and the IgG fraction was separated by precipitation with saturated ammonium sulfate followed by extensive dialysis against phosphate-buffered saline.

2.3. Serum samples

Six yearlings (Horses #1–6) were immunized intramuscularly with two or three doses of inactivated WN vaccine (Innovator, Fort Dodge Animal Health, Fort Dodge, IA, USA) at three-week intervals and bled until 16 weeks after vaccination. Sera collected previously from two yearlings at 28 days after experimental infection (Kitai et al., 2007) were used as a positive control in the conventional ELISA or for comparison in the IgG isotype profiles. For analyzing CDC activities in relation to IgG isotypes, an ICR mouse was immunized repeatedly by intraperitoneal inoculation with inactivated WN vaccine (Innovator, Fort Dodge Animal Health), while infected mouse sera collected previously from two mice 34 days after infection with WNV (Ishikawa et al., 2007) were pooled and used in this study. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University and the Equine Research Institute.

2.4. WNV NS1-expressing cells

The generation of cell lines transfected stably with the NS1 (2G2 cells) or NS1/NS2A genes (2G12 cells) of WNV has been described (Kitai et al., 2007). Briefly, CHO cells were transfected with a pcDNA3-based plasmid expressing the NS1 or NS1/NS2A gene of the WNV Eg101 strain, selected using G418-containing medium, and then cloned by limiting dilution. High percentages (80–100%) of 2G2 or 2G12 cells expressed the NS1 antigen, as determined by immunostaining. 2G2 cells were used as the antigen for the CDC assay, while the culture fluids of 2G2 or 2G12 cells containing secreted NS1 were used as the antigen for the conventional ELISA for detecting antibodies against NS1 of WNV, or the blocking ELISA, respectively.

2.5. Conventional ELISA to measure NS1 antibody levels

Antibody levels to WNV NS1 in horse and mouse sera were measured by a conventional ELISA, essentially following a method described previously (Kitai et al., 2007). Briefly, microplates sensitized with 3 ng of WNV NS1 antigen per well were incubated serially with test sera (1:100 dilution), conjugates and *p*-nitrophenyl phosphate. The WNV NS1 antigen was affinity-purified from culture fluids of 2G2 cells using the monoclonal antibody WN-2H4. The conjugates used in this study were alkaline phosphatase-conjugated rabbit anti-horse IgG (gamma chain-specific; Rockland Immunochemicals, Gilbertsville, PA, USA) and goat anti-mouse IgG (Fc fragment-specific; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). To minimize nonspecific reactions, a non-sensitized control plate was run in parallel, and the difference in absorbances from antigen-sensitized wells was recorded. Serum from a horse at 28 days after experimental infection with WNV (Horse #1; Kitai et al., 2007) was used as a positive control to minimize interplate variations in the ELISA for measuring antibody levels in horses.

2.6. Epitope-blocking ELISA

The epitope-blocking ELISA for differentiating WNV NS1 from JEV NS1 antibodies in horse sera was performed as described previously (Kitai et al., 2007). Briefly, microplates were incubated serially with: (i) rabbit serum hyperimmune to NS1 of WNV; (ii) culture fluids of 2G12 cells; (iii) test sera or ELISA diluent; (iv) WN-2H4 or affinity-purified mouse IgG1 (Bethyl Laboratories, Montgomery, TX, USA); (v) alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories); and (vi) *p*-nitrophenyl phosphate. Test sera were incubated in parallel with the ELISA diluent in step (iii), and WN-2H4 (isotype; IgG1) with mouse IgG1 (without any anti-WNV activity) in step (iv) to minimize nonspecific reactions. The percentage of inhibition of monoclonal antibody binding was calculated from the absorbances using the following formula: $100 - 100 \times [(A - B)/(C - D)]$, where A is the absorbance obtained with a combination of steps (iii) and (iv) with test sera and WN-2H4, B is obtained with the test sera and purified IgG1, C is obtained with ELISA diluent and WN-2H4, and D is obtained with ELISA diluent and purified IgG1, respectively. Sera with inhibition values of 27.6% or greater were determined to be positive for antibodies to WNV NS1.

2.7. CDC assay

The CDC assay for measuring antibodies to WNV NS1 was performed as described previously (Kitai et al., 2010). Briefly, 50 μl of serum-free minimal essential medium (SF-MEM) containing 5×10^4 2G2 cells was mixed with an equal volume of heat-inactivated test serum diluted in SF-MEM and incubated

on ice for 30 min. Then, 11 μl of rabbit complement (Low-Tox-M Rabbit Complement; Cedarlane, Hornby, Canada) were added (final concentration, 10%) and incubated at 37 °C for 2 h. Following centrifugation, 50 μl of the supernatant was mixed with 50 μl of a lactose dehydrogenase (LDH) substrate (Cytotoxicity Detection Kit Plus [LDH]; Roche, Mannheim, Germany) and incubated at room temperature for 15 min, followed by spectrophotometry at 490 nm. The percentage of specific cell lysis was calculated according to the manufacturer's instructions using the following formula: $100 \times [(A - C)/(B - C)]$, where A represents the absorbance obtained with the test serum (experimental release), B represents the absorbance obtained by lysing all of the target cells with 1% Triton X-100 (maximum release), and C represents the absorbance obtained with target cells incubated in SF-MEM containing rabbit complement at 10% (minimum release). The cutoff for specific lysis to differentiate positive from negative sera for WNV NS1 antibodies was 19.8%. In the one-dilution method, the percentage of specific cell lysis obtained in 1:10–1:80 dilutions of sera was used as the WNV NS1 antibody level. In the endpoint method, the WNV NS1 antibody titer was expressed as the highest serum dilution giving greater than 19.8% specific lysis.

2.8. IgG isotyping

The IgG isotypes of WNV-specific antibodies were determined by conventional ELISA, using monoclonal antibodies specific for each equine IgG isotype prepared in our laboratory (Sugiura et al., 1998), commercial horseradish peroxidase-conjugated goat anti-horse IgGa, IgGc or IgG(T) or sheep anti-horse IgGb (Bethyl Laboratories), or alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech, Birmingham, AL, USA). The antibody titer was expressed as the maximum serum dilution showing an absorbance greater than an arbitrary cutoff of 0.2.

2.9. Neutralization test

Neutralizing antibody titers were determined by a plaque reduction assay using the NY99 strain of WNV and Vero cells. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 50% reduction in plaque number.

3. Results

3.1. Time course of WNV NS1 antibodies in sera from horses vaccinated with inactivated WN vaccine

Two groups of three yearlings were immunized with two or three doses of the inactivated WN vaccine and bled until 16 weeks after vaccination (Fig. 1). Prior to evaluation of the blocking ELISA and the CDC assay, sera were tested by the neutralizing test and a conventional ELISA to measure WNV NS1 antibodies (Fig. 1, upper panels). Neutralizing antibody titers began to increase after the second dose in all horses, demonstrating the effectiveness of the vaccine. Overall, three doses induced higher titers than two doses. The time courses of NS1 antibodies paralleled those of neutralizing antibodies in all horses. These results indicated that NS1 antibodies were induced by the inactivated WN vaccine in horses after the second dose, and suggested that NS1 antigen was contained in the vaccine preparation along with E antigen, the main component of the inactivated WN vaccine relevant to the induction of neutralizing antibodies.

3.2. Effect of NS1 antibodies on the blocking ELISA

In the blocking ELISA (Fig. 1, middle panels), the time courses of the inhibition percentages were similar to those obtained using the conventional ELISA. Specifically, all horses became positive after the second dose and remained so until the end of the experimental period (week 16), except for Horse #4 in which the NS1 antibodies (determined by the conventional ELISA) were at the lowest level among the six horses. This result indicated that NS1 antibodies induced by the inactivated WN vaccine were detected by the blocking ELISA.

3.3. Effect of NS1 antibodies on the CDC assay

The vaccine-induced NS1 antibodies were also detected by the end-point method of the CDC assay (Fig. 1, middle panels). However, two of the five horses (excluding Horse #4, which was negative throughout the experimental period) became negative at the end of the experimental period. Furthermore, when sera were tested at a 1:80 dilution in the one-dilution method where specific lysis of 19.8% is set as a cutoff value (Fig. 1, lower panels), almost all samples showed low percentages of specific lysis and only two samples were determined as positive and these were only positive for a limited period after the booster dose (Horse #1 was positive at weeks 6 and 8, and Horse #6 at week 5). These results indicated that although vaccine-induced NS1 antibodies were detectable in the CDC assay, the time period of positive detection was not as long as that shown by the blocking ELISA and was shortest when a 1:80 dilution of serum samples was used in the one-dilution method.

3.4. Comparison between IgG isotypes induced by infection and vaccination in mice and horses

To investigate the mechanism underlying the low NS1 antibody levels shown by the CDC assay, the IgG isotypes of NS1 antibodies induced by the inactivated WN vaccine were analyzed and compared with those induced by infection (Fig. 2A–C). Using a mouse model, the NS1 antibody titers of vaccinated or infected mice were found to be 1:400 or 1:1600, respectively, with an approximately 4-fold lower titer induced by vaccination than by infection, as determined by the conventional ELISA (Fig. 2A, upper panel; open and closed circles). By contrast, the CDC assay provided equivalent percentages of specific lysis (approximately 15%) in vaccinated and infected mice at serum dilutions of 1:100 and 1:1,600, respectively, with a 16-fold difference between vaccinated and infected mice (Fig. 2A, lower panel; open and closed circles). Thus, CDC activity relative to the ELISA antibody level was approximately 4-fold higher in infected than vaccinated mice. For IgG isotyping, ELISA antibody titers obtained with vaccinated and infected mice for each isotype were normalized against those obtained for whole IgG. As shown in Fig. 2B, IgG1 was induced at a higher level by vaccination than by infection, whereas other isotypes (IgG2a, IgG2b and IgG3) showed the opposite profile. Furthermore, a monoclonal antibody of the IgG1 isotype (WN-2H4) showed little CDC activity, in contrast to the high reactivities observed in the conventional ELISA (Fig. 2A; diamonds). These results indicated that the CDC assay showed lower antibody levels in vaccinated than infected mice, and this difference was related to the fact that the IgG1 antibodies predominantly induced by vaccination did not show CDC activity in mice.

Next, the IgG isotypes induced by infection or vaccination were compared in horses (Fig. 2C). Sera selected for this comparison were from Horse #2 at six weeks after the first dose of inactivated WN vaccine, which was obtained in the present study, and from Horse #2 at 28 days after experimental infection with WNV, which was obtained in a previous study (Kitai et al., 2007). These sera

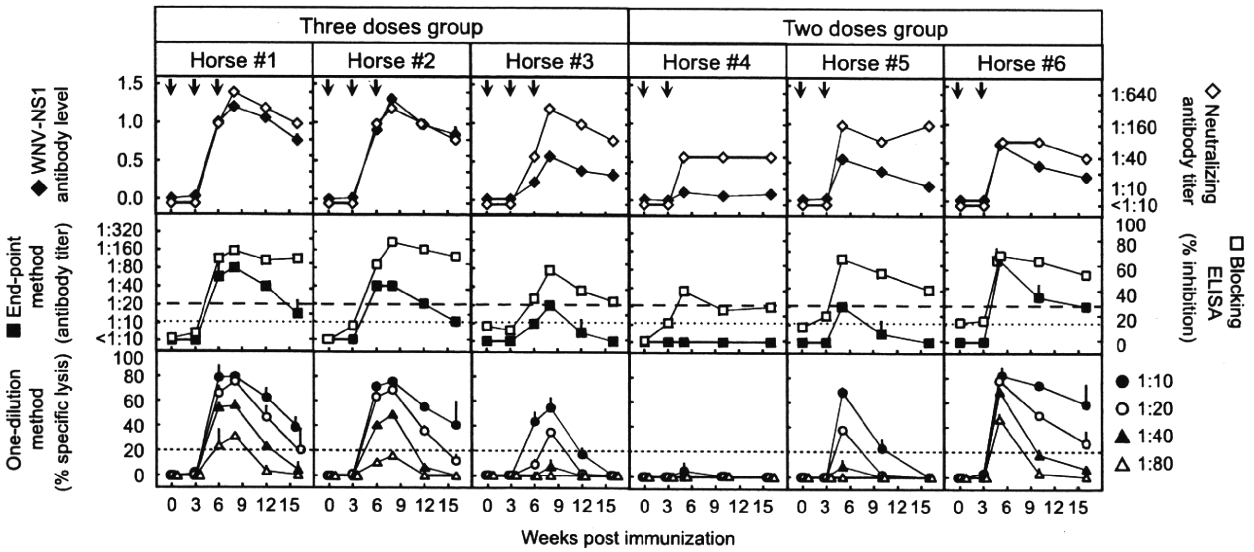

Fig. 1. Time courses of NS1 and neutralizing antibodies in sera serially collected from horses vaccinated with inactivated WN vaccine. Sera from horses immunized with three or two doses of inactivated WN vaccine were tested by the neutralization test (open diamond), conventional ELISA (closed diamond), blocking ELISA (open square), and the CDC assay to measure WNV NS1 antibody. In the CDC assay, sera were tested by the one-dilution method at serum dilutions of 1:10 (closed circles), 1:20 (open circles), 1:40 (closed triangles) and 1:80 (open triangles), and by the end-point method (closed squares). Each datum of the conventional ELISA and CDC assay represents the average value obtained in two separate experiments (standard deviations are indicated by bars). Dotted lines indicate the cutoff values using the one-dilution (19.8%) or end-point (1:10) methods of the CDC assay. Dashed lines indicate the cutoff value in the blocking ELISA (27.6%). Arrows indicate the time of vaccination.

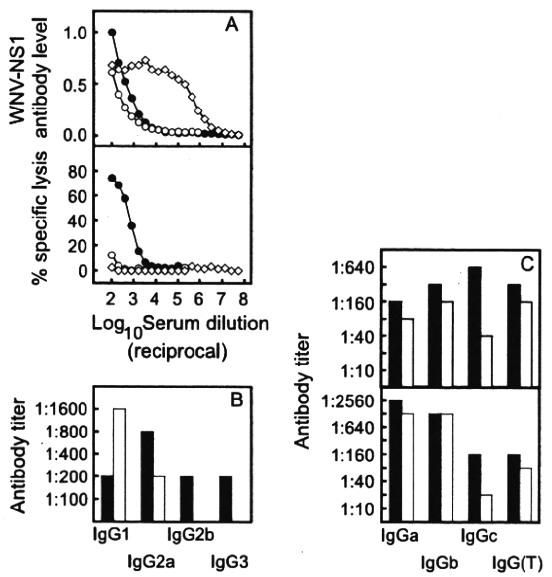

Fig. 2. Isotype analysis of NS1 antibodies induced by vaccination with the inactivated WN vaccine in comparison with those induced by infection. (A) Comparison of the dose–response curves obtained with sera from WNV-infected mice (closed circle), a mouse vaccinated with the inactivated WN vaccine (open circle), and a monoclonal antibody to WNV NS1 (WN-2H4, open diamond), in a conventional ELISA to measure antibodies to WNV NS1 (upper panel) and the CDC assay (lower panel). (B) Comparison of ELISA antibody titers of each IgG isotype in sera from infected (closed bar) or vaccinated (open bar) mice. Titers obtained with infected and vaccinated mice were normalized with antibody titers obtained with whole IgG in the conventional ELISA: the antibody titer of serum pooled from infected mice was 4-fold higher than that of a vaccinated mouse using the conventional ELISA. (C) Comparison of ELISA antibody titers of each IgG isotype induced in infected or vaccinated horses. Sera from an infected horse (Horse #2; Kitai et al., 2007) at 28 days after experimental infection with WNV (closed bar) and from Horse #2 (in the present study) at six weeks after the first vaccination (open bar) were used. Results were obtained using monoclonal antibodies to equine IgG isotypes (upper panel) or commercial conjugates (lower panel) as detector antibodies.

were selected because they showed equivalent ELISA antibody levels (0.993 and 1.101) at a serum dilution of 1:100 and the same ELISA antibody titer (1:800), whereas the geometric mean CDC antibody titers were 1:40 and 1:226 in vaccinated and infected horses, respectively. Two different panels of specific antibodies, in-house monoclonals and commercial polyclonals, were used to detect each isotype in this ELISA for more reliable isotyping of horse IgG. Although IgGa, IgGb and IgG(T) antibodies, which have complement fixing activities, showed lower titers in vaccinated than infected horses for most isotypes, the differences in the isotypes of induced antibodies between vaccinated and infected horses were not as clear as those in mice.

4. Discussion

The findings of this study confirmed those of a previous study by Balasuriya et al. (2006), which reported that inactivated WN vaccine could induce NS1 antibodies in horses: the same vaccine (Innovator, Fort Dodge Animal Health) was used in the present study as had been employed in the study by Balasuriya et al. (2006). The phenomenon was not observed with inactivated JE vaccines that did not induce NS1 antibodies in horses (Konishi et al., 2004). Although attempts to detect NS1 antigens in the inactivated WN vaccine failed (data not shown), it is highly probable that NS1 antigens are present with this vaccine preparation: horses vaccinated with three doses induced higher NS1 antibody levels than those vaccinated with two doses. The process of vaccine preparation probably differs between WN and JE vaccines in terms of the residual NS1 antigens. Although an earlier report showed the presence of NS1 antibodies in horses vaccinated with the inactivated WN vaccine in a particular time point (Balasuriya et al., 2006), the present study investigated the time course of NS1 antibody responses, in relation to their effects on NS1 antibody-based assays.

The ability of the blocking ELISA and the CDC assay to differentiate WNV from JEV infections is based on significant differences in assay results between WNV- and JEV-infected horses, and is therefore disturbed by NS1 antibodies induced in horses vaccinated with inactivated WN vaccine. The present study demonstrated that

inactivated WN vaccine-induced NS1 antibodies were detected by both assays. However, positive results were obtained in a shorter time period in the CDC assay (especially in the one-dilution method using a 1:80 dilution of serum samples) than the blocking ELISA. The 1:80 dilution in the CDC assay is a stringent criterion by which false-positive results by JEV NS1 antibodies are unlikely to be obtained in horses including those vaccinated with inactivated JE vaccine or naturally infected with JEV (Kitai et al., 2010). Using this cutoff point (1:80 dilution), four of the six vaccinated horses were negative for WNV NS1 antibodies throughout the experimental period, while the other two horses were positive for WNV NS1 antibodies for a short period after the booster immunization. On the other hand, horses infected experimentally with WNV that became positive for CDC antibody levels/titers 12–18 days post-infection remained positive until the end of the experimental period (35 days post-infection; Kitai et al., 2010). In addition, quantitative comparisons between the results of the present study and those of a previous study (Kitai et al., 2010) indicated that CDC antibody levels relative to those obtained by a conventional ELISA were lower in vaccinated than infected horses (data not shown). Therefore, the CDC assay is useful for differentiating WNV from JEV infections even in horses vaccinated with the inactivated WN vaccine, provided that this stringent cutoff point (1:80 dilution) is applied.

The IgG isotypes induced and their involvement in CDC activity may be one explanation for the differences in CDC antibody responses induced by infection or vaccination. In general, complement-fixing activities differ between IgG isotypes in mice (Frank and Fries, 1989), humans (Burton and Woof, 1992; Frank and Fries, 1989), or horses (Lewis et al., 2008). Also, the IgG isotype profiles differ between antibodies induced by infection and by protein-based vaccines in horses (Nelson et al., 1998; Sheoran et al., 1997), mice (Simmons et al., 2001) or humans (Giammanco et al., 2003; Huang et al., 2006). In the present study, the relationship between CDC antibody levels and IgG isotypes was shown clearly in a mouse model (Fig. 2A and B). However, isotype analyses in horses did not show differences between infected and vaccinated animals, using sera with equivalent antibody levels in a conventional ELISA (Fig. 2C). The complement fixing activity of equine IgG isotypes remains debatable, since the descriptions in earlier studies with IgGa, IgGb, IgGc and IgG(T) isotypes are different from a recent report that regrouped them as IgG1 to IgG7 (Wagner, 2006; Wagner et al., 2004). Complement-fixing activity has been associated with IgGa and IgGb isotypes according to a report by McGuire et al. (1973). However, IgG(T) may also exhibit this type of activity, since complement-fixing activity is shown by the recently described IgG1, IgG3, IgG4 and IgG7 isotype categories and IgG(T) is related to IgG3 (Lewis et al., 2008). Since horses infected experimentally used in the previous study (Kitai et al., 2007) did not develop any clinical manifestations, symptomatic horses seem to show higher NS1 antibody levels in the CDC assay, providing greater differences in CDC antibody levels from vaccinated horses.

It is assumed that blocking ELISAs based on antibodies to NS1 that are used to differentiate WNV/SLEV (Blitvich et al., 2003) or Kunjin virus/MVEV (Hall et al., 1995) infections are also affected by WNV NS1 antibodies induced by inactivated WN vaccine. In the USA, three WN vaccines for horses, in addition to inactivated vaccines, have been licensed (Kramer et al., 2008). A yellow fever virus (YFV) 17D-based chimeric virus vaccine (Monath, 2001) can induce antibodies to NS1 of YFV, which may cross-react with WNV NS1; however, the canarypox virus-based recombinant and DNA vaccines that lack any flavivirus NS1 gene do not appear to induce antibodies to NS1. In fact, no horses vaccinated with the canarypox virus-based recombinant vaccine induced NS1 antibodies (Balasuriya et al., 2006). In Japan, an inactivated WN vaccine has been licensed but has not yet been employed, since WNV is still absent from Japan. Therefore, for now both assays are useful for differentiating WNV from JEV infections. Under conditions where an inactivated vaccine is in general use, the CDC assay would be a preferable method for differential diagnosis of these viruses.

In conclusion, NS1 antibody-based assays for differentiating WNV from JEV infections are affected by NS1 antibodies induced by inactivated WN vaccine, compromising an effective diagnosis. However, the findings of the present study show that a CDC assay may provide a solution to this problem. A functional assay, like a CDC assay, was found to be less significantly affected by vaccine-induced NS1 antibodies than a binding assay, like a blocking ELISA. Nevertheless, it is still important to confirm the vaccine history prior to differentiation of WNV from other flavivirus infections by NS1 antibody-based assays.

Conflicts of interest

None of the authors have any conflict of interest in relation to the content of the present work.

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Review
Future Virology

Combating Japanese encephalitis: Vero-cell derived inactivated vaccines and the situation in Japan

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Japanese encephalitis (JE) is a major public health threat in Asia, because of its high mortality and high incidence of psychoneurological sequelae in survivors. It is caused by JE virus (JEV) infection, transmitted by vector mosquitoes. The disease is vaccine preventable, and has been well controlled in some countries. Since no specific antivirals have been approved, prevention with vaccine is important in this disease. This article provides a general overview of JE and JEV, but special focus has been put on recently developed Vero cell-derived formalin-inactivated JE vaccines, and the situation in Japan relating to these vaccines. In Japan, where JE has been well controlled, the strong governmental recommendation of the mouse brain-derived vaccine for routine immunization was suspended in 2005, owing to a patient suffering severe postvaccination events. In 2010, the recommendation was reinstated, targeting a limited population utilizing a Vero cell-derived vaccine.

Japanese encephalitis (JE) is an important public health issue; in Asia, it is a major cause of viral encephalitis. The causative agent, JE virus (JEV), a positive-strand RNA virus, is a member of the genus flavivirus in the family *Flaviviridae*. Despite the severe manifestation associated with JEV infection, JE is a vaccine-preventable disease. In fact, countries that have conducted nationwide vaccine programs, such as Japan, Korea and Taiwan, have successfully reduced the number of JE cases since the introduction of JE vaccines (from the 1950s to the 1970s) [1]. However, since internationally available formalin-inactivated vaccines are expensive and require multiple doses, many developing countries in endemic regions face difficulties in implementing nationwide vaccine programs. Therefore, improved vaccines are required, as well as the development of effective antivirals. On the other hand, in areas where JE is well controlled, the necessity of continued vaccination programs has been questioned, since, in these regions the number of mosquito vectors has also reduced. In this article, an overview of the transmission cycle, pathogenesis, geographic distribution and epidemiology of JEV is presented. Then, recently developed antiviral drugs and currently available vaccines, including a Vero cell-derived formalin-inactivated JE vaccine, and the issues surrounding these vaccines, are discussed in detail. In particular, the current status of natural JEV activity amongst vaccinated populations in Japan is considered as a model for reconsidering the necessity for continued vaccination. Specific information about recent advances in JE vaccine development is available elsewhere [2], and is, therefore, not dealt with in this article. A thorough literature search on the subject of antivirals and vaccines was performed using the PubMed and HighWire databases, and findings from those publications are included in this manuscript. In addition, recent domestic publications have been included to address the current status of JE disease control in Japan.

Transmission cycle

Japanese encephalitis virus was first isolated from the brain of a patient in Japan in 1935; this strain was the prototype Nakayama strain. Then, in 1938, JEV was isolated from *Culex tritaeniorhynchus* mosquitoes, again in Japan [3]. More than 30 species of mosquitoes have been shown to be able to transmit JEV, among which *Cx. tritaeniorhynchus* is considered the most important vector for human infection [4,5]. An infective titer of 1–2 $\log_{10}$ per blood meal is sufficient for *Cx. tritaeniorhynchus* to establish a disseminate infection and transmit the virus [6,7]; this corresponds to a viremia level of approximately 10^4–10^5 per ml, supposing that the blood meal size is 2 µl.

Keywords
▪ antivirals ▪ Japanese encephalitis ▪ vaccine