III 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻 号	ページ	出版年
○Eiji Konishi, Yoko Kitai, Yukiko Tabei, Kouichi Nishimura, Seiya Harada	Natural Japanese encephalitis virus infection among humans in west and east Japan shows the need to continue a vaccination program	Vaccine	28 (14)	2664-2670	2010
Yoko Kitai, Takashi Kondo, ○Eiji Konishi	Complement-dependent cytotoxicity assay for differentiating West Nile virus from Japanese encephalitis virus infections in horses	Clinical and Vaccine Immunology	17 (5)	875-878	2010
○Eiji Konishi, Yoko Kitai, Kouichi Nishimura, Seiya Harada	Antibodies to bovine serum albumin in human sera: problems and solutions with casein-based ELISA in the detection of natural Japanese encephalitis virus infections	Jpn J Infect Dis.	63 (4)	296-298	2010
Miwa Kuwahara, ○Eiji Konishi	Evaluation of extracellular subviral particles of dengue virus type 2 and Japanese encephalitis virus produced by Spodoptera frugiperda cells for use as vaccine and diagnostic antigens	Clin Vaccine Immunol.	17 (10)	1560-1566	2010
Jun-ichi Imoto, Tomohiro Ishikawa, Atsushi Yamanaka, Misako Konishi, Kenji Murakami, Tomoyuki Shibahara, Masanori Kubo, Chang Kweng Lim, Masataka Hamano, Tomohiko Takasaki, Ichiro Kurane, Haruhide Udagawa, Yoshihiro Mukuta, ○Eiji Konishi	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	Vaccine	28 (46)	7373-7380	2010
Yoko Kitai, Takashi Kondo and ○Eiji Konishi	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.	J Virol Meth.	171	123-128	2011
Tomohiro Ishikawa, ○Eiji Konishi	Combating Japanese encephalitis: Vero-cell derived inactivated vaccines and the situation in Japan	Future Virology	5(6)	785-799	2010
Tegshduuren E, Yoshimatsu K, Taruishi M, Endo R, Shimizu K, Koma T, Yasuda SP, ○Kariwa H, Arikawa J, Ishihara C	Different cross-reactivity of human and rodentsera to Tula virus and Puumala virus	Comp Immunol Microbiol Infect Dis	33 (6)	e67-e73	2010

Souvik Ghosh, Mohammed Mahbub Alam, Muzahed Uddin Ahmed, Rafiqul Islam Talukdar, Shyamal Kumar Paul and ○Nobumichi Kobayashi	Complete genome constellation of a caprine group A rotavirus srain reveals common evolution with ruminant and huma rotavirus strains	Journal of General Viology	91	2367-2373	2010
Yuan-Hong Wang, ○Nobumichi Kobayashi, Shigeo Nagashima, Xuan Zhou, Souvik Ghosh, Jin-Song Peng. Quan Hu, Dun-Jim Zhou and Zhan-Qiu Yang	Full Genomic Analysis of a Porcine-Bovine Reassortant G4P[6]Rotavisus Stran R479 Isolated from an Infant in China	Journal of Medical Virology	82	1094-1102	2010
Shigeo Nagasima, ○Nobumichi Kobayashi, Shyamal Kumar Paul, Souvik Ghosh, Mamta Chawla-Sarkar, Mohammad Akram Hossain, and Triveni Krisnan	Identification of P[8]b Subtype in OP354-Like Human Rotavirus Strains by a Modidied RT-PCR Method	Jpn. J. Infect. Dis.	63	208-211	2010
Souvik Ghosh, Zipporah Gatheru, james Nyangao, Noriaki Adachi, Noriko Urushibara and ○Nobumichi Kobayashi	Full Genomic Analysis of a G8P[1] Rotabirus Strain Isolated from an Asymptomatic Infant in kenya Provides Evidence for an Artiodactyl-to-Human Interspecies Transmission Event	Journal of Medical Virology	83	367-376	2011
Takashi Matsumoto, Kentaro Yamada, Kazuko Noguchi, Kantou Nakajima, Kenzo Takada, Pakamatz Khawplod, ○Akira Nishizono	Isolation and characterization of novel human monoclonal antibodies possessing neutralizing ability against rabies virus	Microbiol Immunol	54 (11)	673-683	2010
Moazzem Hossain, Tania Bulbul, Kamruddin Ahmed, Ziauddin Ahmed, Mohammad Salimuzzaman, Mohammad Shahidul Haque, Ajmat Ali, Shohrab Hossain, Kentaro Yamada, Kazuhiko Moji, ○Akira Nishizono	Five year (January 2004 – December 2008) surveillance on animal bite and rabies vaccine utilization in the Infectious Disease Hospital, Dhaka, Bangladesh	Vaccine			in press
Takashi Matsumoto, Kamruddin Ahmed, Omala Wimalaratne, Kentaro Yamada, Susilakanthi Nanayakkara, Devika Perera, Dushantha Karunanayake, ○Akira Nishizono	Whole genome analysis of a human rabies virus from Sri Lanka	Arch Virol.			in press

塩田 星児, Kamruddin Ahmed, 三舟 求眞人, ○西園 晃	日本製狂犬病ワクチン皮内接種法による曝露前免疫の有効性の検討	感染症学雑誌	84(1)		2010
Masatani, T, ○Ito, N, Shimizu, K, Ito, Y, Nakagawa, K, Sawaki, Y, Koyama, H, Sugiyama, M	Rabies virus nucleoprotein functions to evade activation of the RIG-I-mediated antiviral response	J Virol.	84(8)	4002-4012	2010
○Ito, N, Moseley, GW, Blondel, D, Shimizu, K, Rowe, CL, Ito, Y, Masatani, T, Nakagawa, K, Jans, DA, Sugiyama, M.	Role of interferon antagonist activity of rabies virus phosphoprotein in viral pathogenicity	J Virol.	84(13)	6699-6710	2010
Masatani, T, ○Ito, N, Shimizu, K, Ito, Y, Nakagawa, K, Abe, M, Yamaoka, S, Sugiyama, M	Amino acids at positions 273 and 394 in rabies virus nucleoprotein are important for both evasion of host RIG-I-mediated antiviral response and pathogenicity	Virus Res.	155	168-174	2011

Ⅳ. 研究成果の刊行物・別刷

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Natural Japanese encephalitis virus infection among humans in west and east Japan shows the need to continue a vaccination program

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ABSTRACT

Japanese encephalitis (JE) is a serious disease in Asia, but it can be prevented by vaccination. To evaluate the necessity for vaccination in areas with reduced numbers of vector mosquitoes, as well as patients, it is critical to understand the frequency of natural virus exposure. An antibody survey was recently conducted to estimate current natural infection rates in Japan, where the vaccination rate has dropped in recent years. Serum samples were collected in 2004–2008 from inhabitants of Kumamoto Prefecture in west Japan, and in 2004–2006 from the Tokyo Metropolitan area of east Japan. Average annual infection rates estimated from the prevalence of antibodies to the nonstructural 1 protein (NS1) of JE virus was 1.8% in Kumamoto and 1.3% in Tokyo. When estimated from percentages of populations with detectable neutralizing antibodies but with no vaccination history, the average annual infection rate was 2.6% in both survey areas. Thus, JE virus remains present and active in nature in Japan. Therefore, continuing a vaccination program is indispensable to prevent JE infection in humans.

1. Introduction

Japanese encephalitis (JE) is a major public health issue in Asia [1]. Annually, approximately 50,000 cases occur and 10,000 die from this disease [2], which is caused by the Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* in the family *Flaviviridae* that is transmitted by mosquitoes [3]. In Japan, Korea and Taiwan, it has been demonstrated that this disease can be controlled by human vaccination [4]. A formalin-inactivated, purified JEV preparation has been widely used to protect human populations from JE.

In Japan, the annual number of human JE cases has been reduced from over 1000 before 1967 to less than 10 after 1992 [5]. This followed the initiation in 1967 of the nationwide distribution of a high-purity inactivated JE vaccine. It has been thought that the reduction in JE cases might also have resulted from the decreased number of vector mosquitoes. In conjunction with the relocation of many pig farms to areas removed from residential zones, such factors would have reduced the efficiency of JEV transmission to human populations from amplifier pigs through vector mosquitoes [6]. Reports on post-vaccination events like acute disseminated encephalomyelitis (ADEM) [7,8] raised opposition to JE vaccination. Then, the occurrence of a case with severe ADEM following JE vaccination prompted the Japanese Government in 2005 to withdraw its strong recommendation for JE vaccination for both 3-dose primary and 2-dose booster immunizations [9,10].

Following the suspension, the JE vaccination rate decreased. The national JE surveillance program indicated that only approximately 10% of children aged 3–4 years were vaccinated with JE vaccine in 2007 [11]. Accordingly, the surveillance program in the same year indicated that only approximately 20% of children of identical ages possessed neutralizing antibodies against JE [12]. Therefore, increasing populations susceptible to JEV infection have raised concerns about the recurrence of JE.

The natural activity of JEV is a critical factor in the debate about the necessity to continue a vaccination program. In Japan, except for the non-endemic northern areas, annual infection rates in humans ranged from 3 to 17% before 1960 [13–16], 5 to 10% in the early 1980s and mid-1990s [17] and 0.2 to 3.4% between 2001 and 2004 [18,19]. Although reports on annual infection rates in humans have not been available since 2004, JEV infection in swine is reported every summer by the national JE surveillance program, particularly in the south and west of Japan [20]. The 2008 surveillance report [21] is shown in Fig. 1.

The present paper reports annual infection rates during 2004–2008 among the inhabitants of Kumamoto Prefecture (west

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Japan) and of the Tokyo Metropolitan area (east Japan) during 2004–2006. Natural infection was detected by measuring antibodies to the nonstructural protein 1 (NS1) of JEV, which allowed the differentiation of infected from vaccinated individuals. Annual infection rates were also obtained from percentages of populations who had no history of JE vaccination but possessed neutralizing antibodies against JEV. Kumamoto and Tokyo are representative of the west and east areas of Japan, since the time-related JEV antibody prevalence in swine in these areas during the epidemic season, as reported from the national JE surveillance program [20,21], are roughly similar to those in their neighboring prefectures.

2. Materials and methods

2.1. Human serum samples

As a part of a national JE surveillance program, sera were collected after signed consent was received from 1190 inhabitants of Kumamoto Prefecture from 2004 through 2008, and 955 inhabitants of the Tokyo Metropolitan area from 2004 through 2006. Upon receiving the consent, inhabitants completed questionnaires including questions about JE vaccination history. For most children, parents were able to refer to a notebook wherein was recorded their child's vaccination history. The period of collecting serum samples was from late August to mid-October in Kumamoto and from July to October in Tokyo. The locations of these survey areas are shown in Fig. 1. Survey subjects ranged in age from 0 to 98 years in the Kumamoto samples and 0 to 76 years in the Tokyo samples. Ages were grouped in 10-year increments, except for those aged over 60 years, which were grouped in one age group (see Supplementary Tables S1 and S2 for age and gender compositions). Serum samples from babies aged <6 months, which may contain maternally transferred antibodies, were not used. All of the Kumamoto samples were examined for both NS1 and neutralizing antibodies, while in the Tokyo samples, a reduced number of samples was used for NS1 antibody testing because of the limited volume of sera remaining (indicated by parenthesis in Supplementary Table S2). The use of all human samples in the present study was approved by the Ethical Committees of the Kobe University School of Medicine and the Tokyo Metropolitan Institute of Public Health.

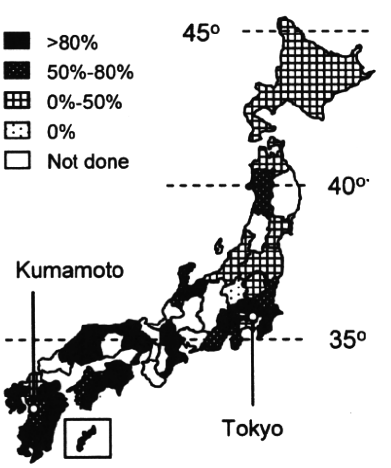

Fig. 1. Map of Japan indicating geographic locations of Kumamoto Prefecture and the Tokyo Metropolitan area and seropositivity in 2008 of the swine population in each prefecture as reported by the national JE surveillance program [21]. Seropositivity of swine is expressed semi-quantitatively in the figure. Note that the surveillance program was not conducted in some prefectures. The island depicted in the square is Okinawa Prefecture, the southernmost prefecture in Japan. Broken lines indicate latitude expressed in degrees north.

2.2. Swine serum samples

As a part of a national JE surveillance program, sera were obtained from 6-month-old swine at slaughterhouses in Kumamoto Prefecture in 2004–2008. Twenty samples were collected on eight occasions during the JE epidemic season from July to September.

2.3. ELISA for quantifying NS1 antibodies

ELISA was performed as described previously [22]. Briefly, plates sensitized with purified NS1 antigens were blocked with the ELISA diluent and then incubated serially with test sera, alkaline phosphatase-conjugated goat anti-human IgG, and *p*-nitrophenyl phosphate. The NS1 antigens were obtained from culture fluids of 3G8 cells stably transfected with the *NS1* and *NS2A* genes of JEV [23] by immunoaffinity purification with a monoclonal specific for NS1 (JE-2D5). The ELISA diluent was composed of 0.05 M Tris–HCl (pH 8.0) containing 0.2% casein, 0.05% Tween 20, 1 mM EDTA and 0.15 M NaCl [24]. The ELISA diluent was used for preparing dilutions of test sera and the conjugate, as well as for blocking. A non-sensitized control plate was run in parallel, and the absorbance obtained with non-sensitized wells was subtracted from those obtained with antigen-sensitized wells, to eliminate nonspecific reactions. To minimize interplate variations, a constant positive control serum was included in every plate, and absorbances obtained with test samples were adjusted with the value for the positive control as 1.0. The adjusted absorbances were expressed as ELISA values. ELISA values of 0.185 or higher were determined as positive for NS1 antibodies.

2.4. Neutralization test

Neutralizing antibodies contained in human sera were titrated by a standard method as described elsewhere [25], as a part of a national JE surveillance program. In this method, complement was not included in the virus–antibody mixture. The neutralizing antibody titer was expressed as the highest serum dilution yielding a 50% reduction in plaque number. Serum samples showing neutralizing antibody titers of 1:10 or higher were determined to be positive for antibodies to JEV.

2.5. Hemagglutination-inhibition (HAI) test

HAI test was performed by a micro-modification of the method of Clarke and Casals [26], with four hemagglutinin units of the JEV antigen (JaGAr#01 strain; Denka Seiken, Niigata, Japan). Sera showing HAI antibody titers of 1:10 or higher were determined to be positive for JEV antibodies.

2.6. Statistical analysis

Significant differences in antibody prevalence were evaluated by the chi-square test with the Yates' correction factor. Probability levels (P) of less than 0.05 were considered significant.

3. Results

3.1. NS1 antibody levels in the Kumamoto populations

Sera collected in Kumamoto Prefecture were examined for NS1 antibodies (Fig. 2A). The overall prevalence of NS1 antibodies in 2004–2008 was 7.6% (90/1190). Males and females did not show significant differences in overall prevalence in each year. However, when comparisons were made among each age group, males showed a higher prevalence than females in the 60s age group in

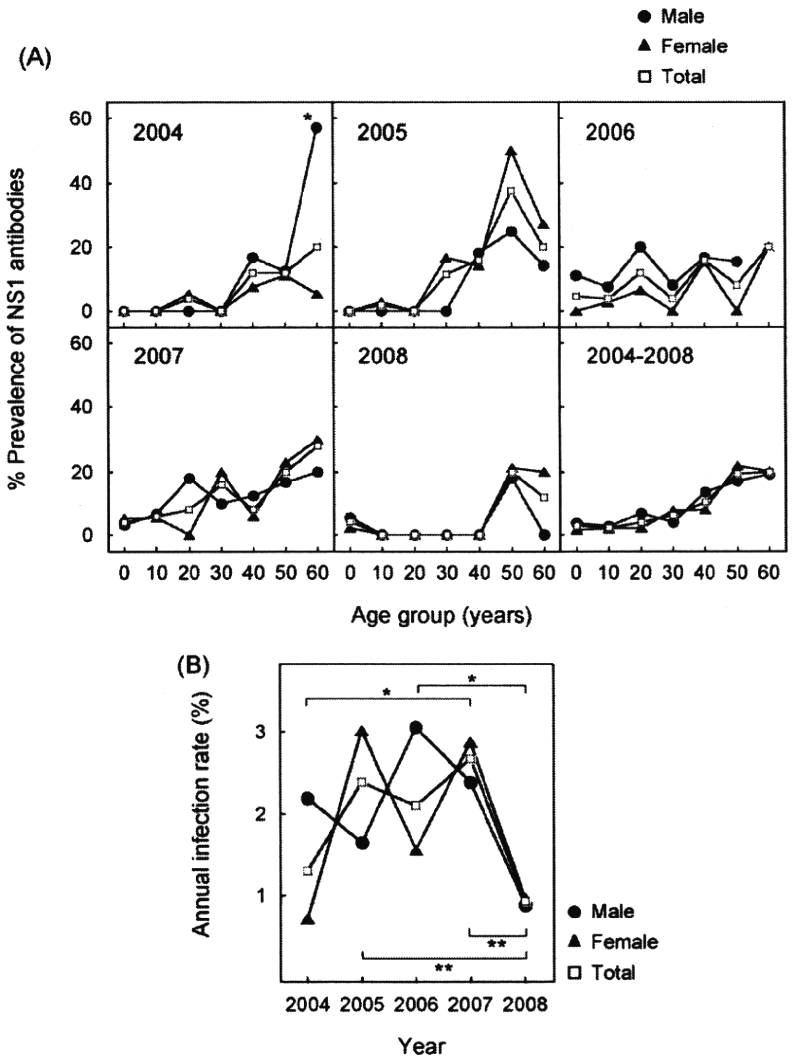

Fig. 2. NS1 antibody prevalence in Kumamoto populations in 2004–2008. (A) Age-dependent antibody prevalence curve in males (closed circle), females (closed triangle) and total (open square) populations. Asterisk indicates significant differences between males and females ($P < 0.05$). (B) Annual infection rates estimated from NS1 antibody prevalence in males (closed circle), females (closed triangle) and total (open square) populations. Asterisks indicate significant differences between years in the total population: *$P < 0.05$ and **$P < 0.01$.

2004. Antibody prevalence increased with age: in the total population (2004–2008), the age groups of the 50s or the 60s showed higher prevalences than those of the 0s, 10s or 20s ($P < 0.001$) or the 30s ($P < 0.01$) and; the 40s age group showed a higher prevalence than did the 0s or 10s ($P < 0.01$).

The annual infection rate was calculated by dividing the NS1 antibody prevalence by the duration of NS1 antibodies (4.2 years [22]). The mean annual infection rate obtained in 2004–2008 was 1.9% in males, 1.7% in females and 1.8% in the total population without significant gender differences ($P > 0.05$). The annual infection rates in 2005–2007 were higher than those in 2004 and 2008: significant differences were detected between 2004 and 2007 and between 2008 and each year for 2005–2007 ($P < 0.05$ or $P < 0.01$; Fig. 2B).

3.2. Neutralizing antibody titers in the Kumamoto populations

The prevalence of neutralizing antibodies was obtained using the same populations as used for the NS1 antibody survey (Fig. 3). Although age-dependent prevalence curves varied between males and females probably based on the small population size in each age group in different years, the total population (2004–2008) showed a tendency that the prevalence increased between the 0s and 10s, decreased between the 20s and 40s and again increased between the 40s and 60s age groups. The overall prevalence for 2008 (55.8%) was lower than those for 2004 (66.4%; $P < 0.05$) and 2005 (70.5%; $P < 0.001$). Antibody prevalences in the 0s age group in 2007–2008 were lower than those in 2004–2006 ($P < 0.05$).

Questionnaires related to the vaccination history of each subject provided a total of 145 children aged 9 years or younger who had not received any JE vaccine (Table 1). Of these, 15 (10.3%) were positive for neutralizing antibodies, indicating natural infection with JEV. Since the average survival period of these subjects was 4.0 years, the annual infection rate was calculated to be 2.6% (Table 1). The annual infection rates in males and females obtained in the same way were 2.4 and 2.9%, respectively.

Prevalence of HAI antibodies among pigs started to increase in late July in 2005 and 2007 and in late August in other years (Fig. 4). The time when the prevalence became over 50% was the latest in 2008 (mid-September). These results suggested that the

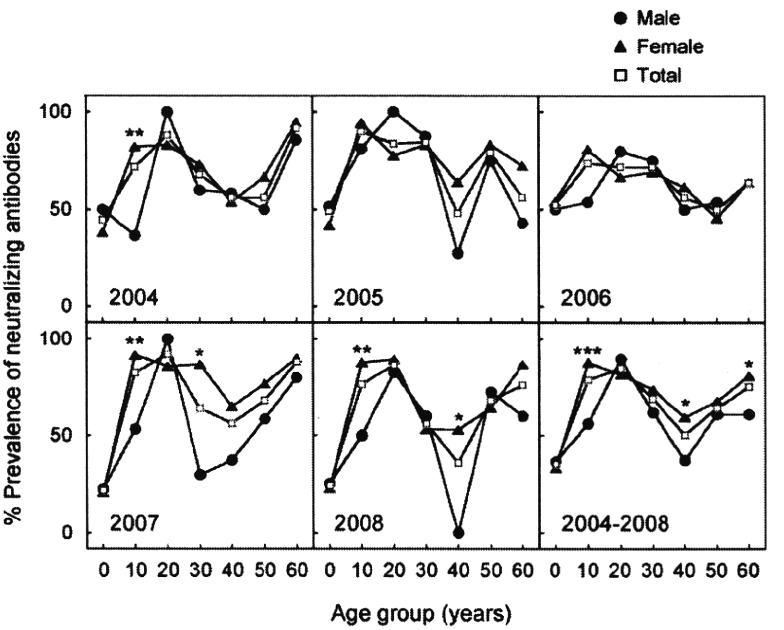

Fig. 3. Neutralizing antibodies in Kumamoto populations in 2004–2008. Age-dependent antibody prevalence was obtained in males (closed circle), females (closed triangle) and total (open square) populations. Asterisks indicate significant differences between males and females: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table 1
Annual infection rates in Kumamoto, 2004–2008, and Tokyo, 2004–2006, calculated from the number of unvaccinated children aged 0–9 years with neutralizing antibodies.

Area	Gender	Total no.	No. of positive	% positive	Average survival period (year)[a]	Annual infection rate (%)[b]
Kumamoto	Male	80	7	8.8	3.7	2.4
	Female	65	8	12.3	4.3	2.9
	Total	145	15	10.3	4.0	2.6
Tokyo	Male	127	13	10.2	3.2	3.2
	Female	73	3	4.1	2.8	1.5
	Total	200	16	8.0	3.1	2.6

[a] Calculated from the age of the subjects. The survival period of each subject was supposed to be 0.5 years more than the age: for instance, the survival period of a subject aged 1 year was regarded as 1.5 years.
[b] Calculated by dividing the "% positive" by the "average survival period".

spread of JEV was earlier in 2005 and 2007 than in other years and that JEV activity in 2008 was the lowest for the 2004–2008 period in Kumamoto, as far as the activity could be estimated by swine antibodies.

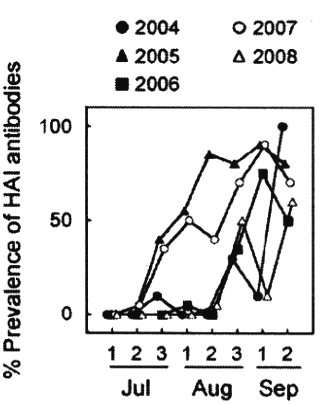

Fig. 4. Time-related prevalence of HAI antibodies in swine in Kumamoto Prefecture. Sera were collected in early (1), mid (2) and late (3) July, August and September in 2004 (closed circle), 2005 (closed triangle), 2006 (closed square), 2007 (open circle) and 2008 (open triangle).

Finally, we examined the relationship between neutralizing and NS1 antibodies. Overall, 6.4% of the population were positive for both neutralizing and NS1 antibodies, 57.1% were positive only for neutralizing and 1.2% were positive only for NS1 antibodies, while 35.4% were negative for both antibodies. By age-dependent curves (Fig. 5), approximately 80% of the 10s and 20s populations had only neutralizing antibodies. As well, approximately 20% of the 50s and 60s were positive for both neutralizing and NS1 antibodies, in contrast to less than 5% of the 30s or lower age groups.

3.3. NS1 antibody levels in the Tokyo populations

NS1 antibodies in sera collected in the Tokyo Metropolitan area were analyzed in a similar way to that used for the Kumamoto populations (Fig. 6A). Overall prevalence of NS1 antibodies in 2004–2006 was 5.5% (32/578). No significant differences were shown in the prevalence between males and females in each age group in each year ($P>0.05$). Similar to the Kumamoto populations, prevalence increased with age. Among the total population (2004–2006), the 50s and 60s age groups showed higher prevalences than the 10s ($P<0.001$) or 20s ($P<0.01$), while the 50s age group showed a higher prevalence than that the 0s ($P<0.05$).

The mean annual infection rates calculated from NS1 antibody prevalences in 2004–2006 were 1.0% in males, 1.6% in females and 1.3% in the total population without significant gender differences

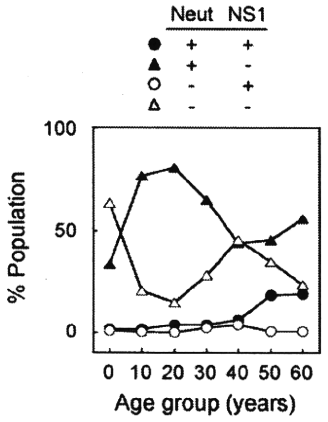

Fig. 5. Relationships between neutralizing and NS1 antibodies in Kumamoto populations. Age-dependent percentages were obtained from populations who were positive for both neutralizing and NS1 antibodies (closed circle), positive only for neutralizing antibodies (closed triangle), positive only for NS1 antibodies (open circle), and negative for both antibodies (open triangle).

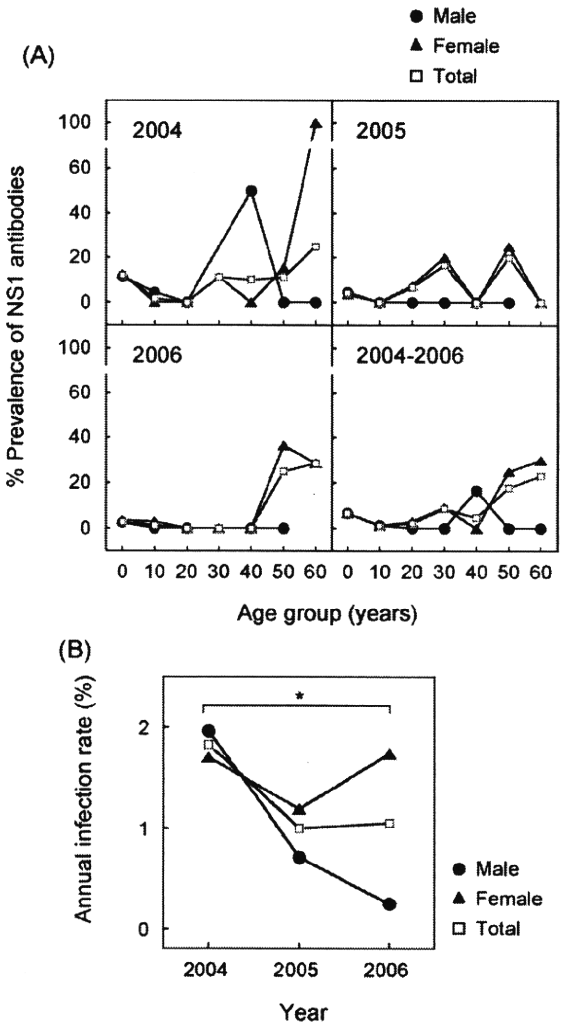

Fig. 6. NS1 antibody prevalence in Tokyo populations in 2004–2006. (A) Age-dependent antibody prevalence curve in males (closed circle), females (closed triangle) and total (open square) populations. (B) Annual infection rates estimated from NS1 antibody prevalence in males (closed circle), females (closed triangle) and total (open square) populations. Asterisk indicates a significant difference between years in the male population ($P < 0.05$).

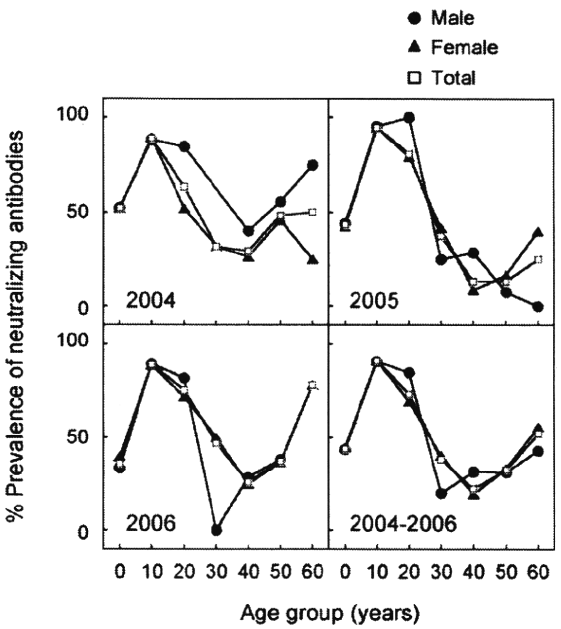

Fig. 7. Neutralizing antibodies in Tokyo populations in 2004–2006. Age-dependent antibody prevalence was obtained in males (closed circle), females (closed triangle) and total (open square) populations.

($P > 0.05$). The annual infection rates were not significantly different for the years 2004–2006, except for in the male population where the rate was significantly higher in 2004 than 2006 ($P < 0.05$; Fig. 6B).

3.4. Neutralizing antibody titers in the Tokyo populations

Age-dependent prevalence curves of neutralizing antibodies (Fig. 7) showed a tendency similar to those shown in the Kumamoto populations (Fig. 3): a low prevalence among the 0s years, high prevalences for the 10s and 20s and a low prevalence for the 40s age groups in the total population (2004–2006). The prevalence of neutralizing antibodies was not significantly different between genders in each year and each age group ($P > 0.05$; Fig. 7). On the other hand, the prevalence was significantly lower in 2006 than 2004 in the 0s age group in the male and total populations ($P < 0.05$).

The annual infection rate calculated from the number of children aged 9 years or younger who had not received any doses of JE vaccine was 3.2% in males, 1.5% in females and 2.6% for the total population (Table 1).

4. Discussion

The present survey estimated annual infection rates using two types of antibodies, those to JEV structural or those to nonstructural proteins. Because most people among the total Japanese population have received inactivated JE vaccine in their childhood, identification of naturally infected individuals required a serological method based on antibodies to nonstructural proteins induced only by infection. On the other hand, the recent reduction in vaccination rates allowed us to detect natural infection using a conventional neutralization test. If an unvaccinated individual possessed neutralizing antibodies, we considered that it was as a result of infection. In the present study, the population used for this estimation was limited to those aged 9 years or younger: the information for vaccination history is more reliable in younger than in older people since the objects of the JE vaccination program were

children aged 3–15 years before May of 2005 and 3–10 years after June of 2005.

The annual infection rates obtained based on neutralizing antibodies (2.6% in Kumamoto and Tokyo) were higher than those based on NS1 antibodies (1.8% in Kumamoto and 1.3% in Tokyo). The cutoff value differentiating positive from negative samples in ELISA for measuring NS1 antibodies was determined by the confidence limit calculated from the mean and standard deviation obtained with negative controls at a probability level of 0.1% [22]: thus, theoretical false-positives may arise in only one of a thousand negative samples. On the other hand, the neutralization test was based on a 50% plaque reduction method and the cutoff value was an antibody titer of 1:10, which cannot be considered a stringent cutoff value since a recent clinical trial for evaluating an inactivated JE vaccine used a cutoff of 1:20 for selecting uninfected volunteers [27]. However, in the present study, even if the cutoff was increased to 1:20, the annual infection rate was identical in Kumamoto populations and 2.3% in Tokyo populations. Vaccine-induced "sterile" immunity in which neutralizing antibodies inactivate the virus before infecting the host cells is also a potential reason why the annual infection rates estimated from neutralizing antibodies were higher than those estimated from NS1 antibodies. Although annual infection rates estimated by the two methods varied from 1.3 to 2.6%, this approach provided strong evidence of recent natural JEV activity in Japan.

The national JE surveillance program has reported higher natural JEV activities in Kumamoto Prefecture than in the Tokyo Metropolitan area [20,21], with HAI antibody prevalence among swine during 2004–2008 averaging 59% in Kumamoto and 18% in Tokyo. This is consistent with the difference between the numbers of JE patients reported during the same period in Kumamoto (6 cases) and in Tokyo (0 cases) [28,29]: of particular note was a 3-year-old unvaccinated patient reported in Kumamoto Prefecture in 2006. On the other hand, the difference in NS1 antibody prevalence between Kumamoto in 2004–2008 (7.6%) and Tokyo in 2004–2006 (5.5%) was not statistically significant ($P > 0.05$). Moreover, the annual infection rates estimated from the prevalence of neutralizing antibodies were identical (2.6%). Variations could have occurred in the survey results in both areas, since the survey population was small: 0.012–0.017% and 0.0024–0.0026% of the total population in Kumamoto Prefecture (1,842,140 people) and the Tokyo Metropolitan area (12,570,904 people), respectively, based on the 2005 census. However, the presence of NS1 antibodies and the presence of neutralizing antibodies in unvaccinated children undoubtedly demonstrated natural JEV activities during 2004–2008 in Kumamoto and 2004–2006 in Tokyo. In fact, 2 of the 3 patients in 2008 occurred in east Japan close to Tokyo [29].

In Kumamoto, the annual infection rates estimated from NS1 antibodies were higher in 2005–2007 (2.1–2.7%) than in 2004 (1.3%) or 2008 (0.9%). This appeared consistent with the start of the circulation of JEV in nature, which was earlier in 2005 and 2007 than in other years, as determined by the time course of the prevalence of HAI antibody in swine. However, considering the variation in annual infection rates based on the period for collecting serum samples (from late August to mid-October) and the duration of NS1 antibodies (4.2 years), it cannot always be taken to mean that the human NS1 antibody results correlated with those for swine HAI antibodies.

The age-dependent prevalence of neutralizing antibodies showed similar patterns in Kumamoto and Tokyo. These patterns were consistent with the pattern reported from the national JE surveillance program: that is, an increase between the 0s and 10s, a decrease between the 20s and 40s and then an increase between the 40s and 60s age groups [12]. The low prevalence in the 0s age group reflected a recent decrease in the vaccination rate. The prevalence in this age group was not as high as those reported in 2004 by the national JE surveillance program [28]. During the present survey period, the prevalence in the 0s age group was significantly reduced in both Kumamoto and Tokyo. By contrast, the high percentages in the 10s and 20s age groups are considered as the result of the high vaccination rates before 2004. The low percentage in middle age groups can probably be attributed to the duration of the effect of inactivated JE vaccine and the assumption that almost none received a vaccination after they were 15 years old. The high prevalence rates in the older age groups may be explained by the fact that these ages did not receive vaccinations and were exposed to the JEV antigen initially by natural infection, which may induce stronger memory immune responses than those induced by vaccination.

The relationship between neutralizing and NS1 antibodies varied with age. Since the strategies to measure neutralizing or NS1 antibodies are fundamentally different in terms of the assay method (functional or binding assays, respectively), as well as the type of target protein (structural or nonstructural proteins, respectively), these antibodies do not always appear to correlate. Specifically, an individual who was vaccinated but not infected may develop only neutralizing but not NS1 antibodies. That a high percentage (57.1%) of the population is positive only for neutralizing antibodies (Fig. 5) seems to be attributable to the vaccination. The fact that the populations positive for both neutralizing and NS1 antibodies were higher among the 50s and 60s than in other age groups is related to the present age-dependent curves of NS1 and neutralizing antibodies; with higher prevalences shown in both populations. The high prevalence of NS1 antibodies in the 50s and 60s age groups in Kumamoto and Tokyo populations may be related to differences in the first exposure to a JEV antigen (infection or vaccination) and/or simply how many times an individual has acquired natural infections.

A question arises why the numbers of reported JE patients have stayed low in situations where a relatively large number of unvaccinated children have been exposed to natural JEV infection. One possible explanation is the attenuation of JEV currently circulating in Japan. It is speculated that the attenuation may relate to the recent genotype shift from type 3 to 1 [30] and/or the deletion of about 10 nucleotides in the 3′-untranslated region in recent isolates [31]. Attenuation of JEV is considered to decrease the clinical:subclinical infection rate, resulting in high antibody prevalence with few patients. In recent years, JEV sequences have been detected by polymerase chain reaction in cerebrospinal fluid samples from children with aseptic meningitis in Japan [32]. Attenuation may have caused a shift in the major clinical manifestation caused by JEV infection from encephalitis to meningitis, which would have contributed to the reduction in the numbers of reported JE patients.

In conclusion, the present survey, conducted in areas of west and east Japan, revealed continuous JEV activities in nature and recent exposures of human populations to JEV infection. This indicates that the JEV transmission route to humans still exists in domestic and peridomestic environments in Japan. Although the wild JEV strain might be currently attenuated, it is possible that the virus could recover virulence through natural mutation and foreign pathogenic JEV strains could invade Japan [33]. These results highlight the necessity for the National Government to again strongly recommend JE vaccination and prevent JE infections in Japan's population.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.01.008.

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Complement-Dependent Cytotoxicity Assay for Differentiating West Nile Virus from Japanese Encephalitis Virus Infections in Horses[▽]

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A complement-dependent cytotoxicity (CDC) assay was established to measure antibodies to the West Nile virus (WNV) nonstructural protein 1 (NS1) in horses. Sera collected from a WNV-infected horse mediated lysis of WNV NS1-expressing cells in a dose-dependent manner at higher percentages than sera from a Japanese encephalitis virus (JEV)-infected horse. The percentages of specific lysis for sera diluted 1:10 to 1:80 were <19.8% (assay cutoff) for almost all of the 100 JEV-infected or uninfected horses tested, in contrast to 55 to 76% in WNV-infected horses. Experimental infection revealed that horses became anti-WNV NS1 antibody positive 10 days after WNV infection. This study demonstrated the utility of this assay for differentiating WNV from JEV infections in horses.

West Nile virus (WNV) and *Japanese encephalitis virus* (JEV) are included in the Japanese encephalitis (JE) serological group of the *Flavivirus* genus of the *Flaviviridae* family (4, 13). JEV is distributed mostly in Asia, whereas WNV is spread worldwide, apart from most areas of Asia (3, 14, 17). There is a possibility that WNV may be introduced into Asia including Japan, similar to its introduction and rapid expansion in the Western Hemisphere. Since the clinical features of WNV infection in humans and horses (6, 16) are similar to those caused by JEV (2, 16), the differential diagnosis of WNV from JEV infections is reliant on laboratory tests. The neutralization test provides the highest specificity among currently available serodiagnostic tests (11). However, even with the neutralization test, cross-reactivity among members of the JE serological group can affect a differential diagnosis, making it difficult to differentiate between WNV and JEV infections (11). Experiments using mice (12), pigs (18), and horses (15) have indicated that upon infection with WNV, animals preimmune to JEV by vaccination or infection induce strong anamnestic responses to JEV. Specifically, neutralizing antibody levels against JEV are commonly equivalent to or even higher than those against WNV.

Antibody-mediated complement-dependent cytotoxicity (CDC) is a mechanism whereby complement activation triggered by specific antibody binding to an antigen on a cell surface causes the formation of the C5b-9 membrane attack complex, which can lyse the target cell (1). We have previously shown the usefulness of this mechanism for measuring antibodies to the nonstructural protein 1 (NS1) of JEV in equine sera (7). In the present study, we examined if the CDC assay could be applied for the detection of antibodies to NS1 of WNV and if this was able to differentiate WNV from JEV infections in horses.

Sera obtained from horses experimentally infected with WNV have been described previously (5). Briefly, two 1-year-old thoroughbred horses (yearlings; horse numbers 1 and 2) were infected subcutaneously with 1×10^7 PFU of the NY99 strain of WNV. Serum collected from horse 1 at 28 days postinfection was used as a positive control in the present CDC assay and the conventional enzyme-linked immunosorbent assay (ELISA). Sera from 100 individual thoroughbred horses born and kept in Japan were used as negative controls for antibodies to WNV NS1, as in our previous study (5). Of the 100 sera, 40 were negative and 60 positive for anti-JEV NS1 antibodies as determined by ELISA (8). The 40 sera that were negative for JEV NS1 antibodies were collected from 20 yearlings vaccinated with inactivated JE vaccine and 20 without vaccination. All 40 of these horses were born and kept in an area of northern Japan where JEV is not endemic. The 60 sera positive for JEV NS1 antibodies were collected from horses aged 3 to 12 years, as used in our earlier survey (9). All animal experiments were conducted according to the Guidelines for Animal Experimentation at the Equine Research Institute.

The CDC assay previously established for measuring JEV NS1 antibodies (7) was modified to detect WNV NS1 antibodies. The antigen used for the present assay was a stably transfected 2G2 cell line that constitutively expresses the NS1 protein of the WNV Eg101 strain (5). Fifty microliters of serum-free minimal essential medium (SF-MEM) containing 5×10^4 2G2 recombinant cells was mixed with an equal volume of heat-inactivated test serum diluted in SF-MEM and incubated on ice for 30 min. This mixture was then mixed with 11 μl of commercial rabbit complement (Low-Tox-M rabbit complement; Cedarlane, Hornby, Canada) for a final concentration of 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; Roche, Mannheim, Germany) and incubated at room temperature for 15 min. The resulting color reaction was

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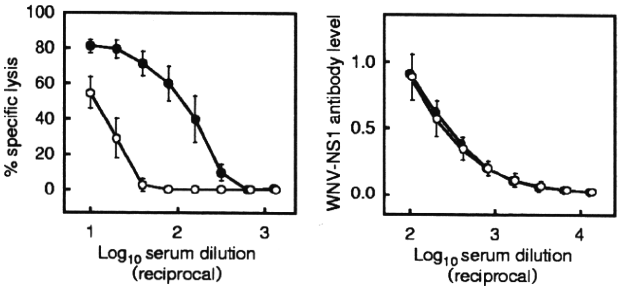

FIG. 1. Comparison of dose-dependent antibody responses of the CDC assay and ELISA to detect anti-WNV NS1 antibodies. Sera from horses experimentally infected with WNV (horse number 1 at 28 days after infection; closed circles) or naturally infected with JEV (open circles) were tested for WNV NS1 antibodies by the CDC assay (left panel) and a conventional ELISA (right panel). A conventional ELISA was performed essentially following a method previously described (5) except that 3 ng of WNV NS1 antigen from culture fluid of 2G2 cells per well was used in this study. Serial 2-fold dilutions of sera starting from 1:10 or 1:100 dilutions were used in the CDC assay or conventional ELISA, respectively. Each datum represents an average obtained in two separate experiments (standard deviations are indicated by bars).

read by spectrophotometry at 490 nm. All procedures were performed in duplicate in 96-well microplates. 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 value obtained with 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). When this calculation provided a negative value, 0.0% was assigned as the result. Test sera with a specific lysis percentage greater than the cutoff value (19.8% of specific lysis) were determined to be positive for WNV NS1 antibodies. For the one-dilution method, the percentages of specific cell lysis obtained in 1:10 to 1:80 dilution series of sera were used as the WNV NS1 antibody level. In the end point method, the WNV NS1 antibody titer was expressed as the highest serum dilution giving greater than 19.8% of specific lysis.

The dose-response curve obtained in the present CDC assay using serum from WNV-infected horses and WNV NS1-expressing cells (Fig. 1, left panel, closed circles) was consistent with previous reports using serum from JEV-infected horses and JEV NS1-expressing cells (7). This provided validation of the CDC assay for measuring WNV NS1 antibodies in horse sera. Percentages of specific lysis were higher than those obtained with serum from JEV-infected horses at most serum dilutions, although two sera showed equivalent WNV NS1 antibody levels in the ELISA (Fig. 1, right panel).

The usefulness of the present CDC assay for differentiating WNV from JEV infections was determined using a total of 100 samples collected from individual thoroughbred horses regarded as free of antibodies to WNV (Fig. 2A). Serum dilutions of 1:10 to 1:80 showed that most of these sera had less than 20% specific lysis, which was lower than the 54.5 to 75.5% levels obtained with the positive control serum (horse 1 at 28

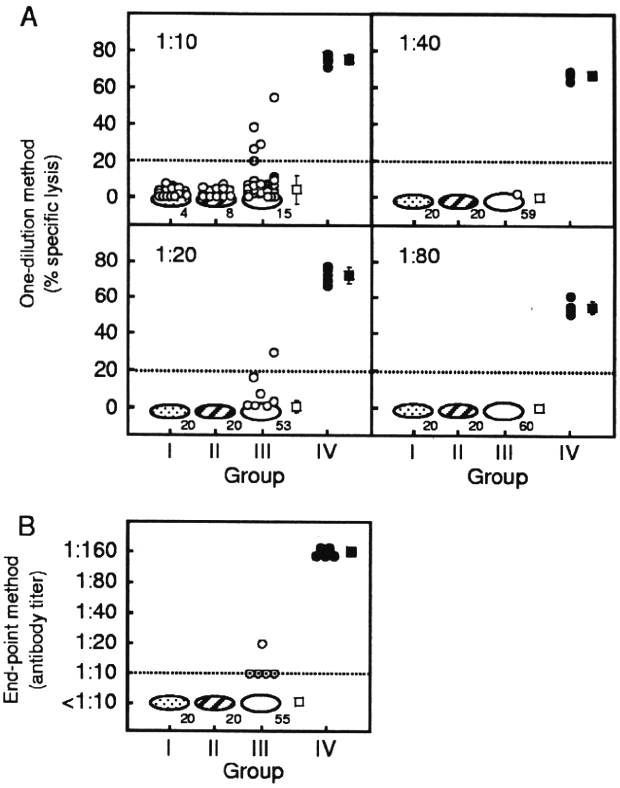

FIG. 2. Distribution of CDC antibody levels/titers in horse sera regarded as negative for WNV NS1 antibodies and sera from a WNV-infected horse. (A) CDC antibody levels obtained by the one-dilution method. Four 2-fold dilutions of sera, 1:10 to 1:80, were used. (B) CDC antibody titers obtained with the end point method. Four groups used for comparison included samples from 20 yearlings without JE vaccination (group I), 20 yearlings with JE vaccination (group II), 60 horses positive for JEV NS1 antibodies (group III), and horse number 1 at 28 days after experimental infection with WNV (group IV). Data of group IV were obtained from five independent experiments. Circles indicate individual data, while ellipses indicate a group of data showing 0.0% specific lysis or an antibody titer of <1:10. The number of individuals contained in the ellipses is given below each ellipse. For groups I to III or IV, open or closed squares with bars indicate averages and standard deviations, respectively. Dotted lines indicate the cutoff value for the one-dilution (19.8%) or end point (1:10) method of the CDC assay.

days after infection) in five separate experiments. We determined that 19.8% was an appropriate cutoff value to differentiate positive from negative samples for antibodies to WNV NS1. This value was calculated by the mean (4.08%) plus 2 times the standard deviation (7.85%) of the percentage of specific lysis obtained at the 1:10 dilution. Under this cutoff, all of the 40 horses without antibodies to JEV NS1 were negative for WNV NS1 antibodies, even in a 1:10 dilution of sera, irrespective of whether these horses were vaccinated. Of the 60 horses with antibodies to JEV NS1, only 5 were positive at a 1:10 dilution and 1 at 1:20; there were no positive sera at 1:40 and 1:80 dilutions. The end point method, which overcomes the limitations of the one-dilution method, which cannot correctly measure high antibody levels (Fig. 2B), indicated that there were antibody titers of <1:10 in 95 of 100 negative samples, in contrast to 1:160 in the positive control. These

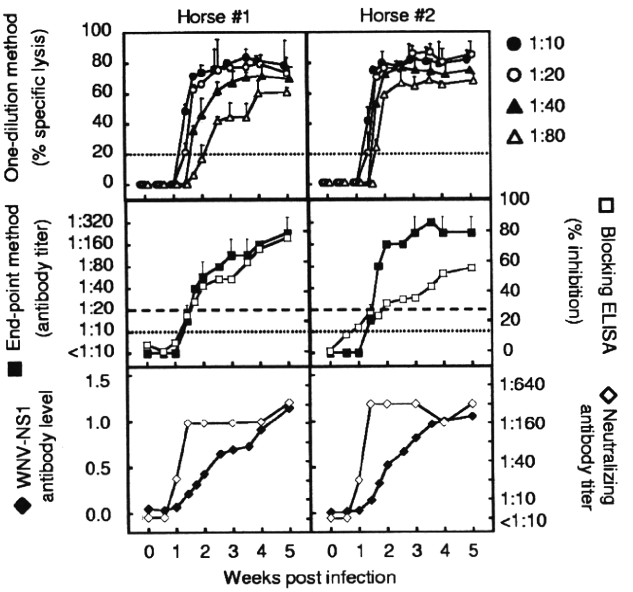

FIG. 3. Time courses of WNV NS1 antibodies in two horses experimentally infected with WNV. Sera were tested by the one-dilution method at serum dilutions of 1:10, 1:20, 1:40, and 1:80 and the end point method of the CDC assay. For comparison, data of the blocking ELISA, conventional ELISA to measure WNV NS1 antibodies, and neutralizing test were included from our earlier study (5). Each datum of the CDC assay represents an average obtained in two separate experiments (standard deviations are indicated by bars). Dotted lines indicate the cutoff values for the one-dilution (19.8%) or the end point (1:10) method of the CDC assay. Dashed lines indicate the cutoff value in the blocking ELISA (27.6%). The week zero time point on the abscissa indicates the sample taken immediately before the WNV infection.

results indicated a clear difference between sera from WNV- and JEV-infected horses in the present CDC assay, as well as the reproducibility of this assay.

To further evaluate the usefulness of the CDC assay, we tested sera from two horses experimentally infected with WNV (Fig. 3). For comparison, time courses of antibody levels/titers previously obtained in the conventional ELISA, the blocking ELISA, and neutralization test (5) are included in Fig. 3. With the one-dilution method of the CDC assay, percentages of specific lysis obtained in a 1:10 dilution of serum started increasing on day 10 and then plateaued at approximately 70 to 80% on day 12 for both horses. Even at serum dilutions of 1:20 to 1:80, both horses became positive within 18 days after WNV infection. The time courses obtained by the end point method were essentially consistent with those obtained by the one-dilution method. In the blocking ELISA, the percentage of inhibition started increasing on day 10, identical to the time course shown in the CDC assay. However, seroconversion was detected on days 12 (horse 1) and 14 (horse 2) by the blocking ELISA, which was slightly later than determined by the CDC assay (day 10). The conventional ELISA first detected WNV NS1 antibodies on day 10, consistent with the time point when antibody levels were shown to increase in the CDC assay and blocking ELISA. The neutralization titers, mainly represented by antibodies to the WNV envelope (E) protein, began to increase on day 7 and then plateaued on day 10, earlier than the increase in WNV NS1 antibodies detected by other methods. These results indicated that the present CDC assay can measure antibodies to WNV NS1 in horses, similar to the blocking ELISA.

In an earlier study using a JEV model (7), we characterized the CDC assay as a method for antibody testing and showed that such an assay can differentiate antigen-specific from nonspecific reactions more effectively than ELISA. Specifically, almost all sera from nonimmune animals at a 1:10 dilution showed no nonspecific lysis using MF6 cells expressing JEV NS1 protein. Similarly, none of the 40 sera we regarded as free from WNV NS1 antibodies and also not containing antibodies to JEV NS1 showed specific lysis (0.0%) at a 1:20 dilution using 2G2 cells expressing WNV NS1 protein. Since 28 of the 40 sera showed low-level lysis (0.17 to 7.48%) at a 1:10 dilution, the ability of the CDC assay to differentiate antigen-specific from nonspecific reactions seems, to some extent, to differ according to the cell line.

The ability of the CDC assay to measure virus-specific antibodies more effectively than cross-reactive antibodies in a model of WNV and JEV is supported by a recent report indicating that JEV NS1 antibodies do not mediate CDC of WNV-infected cells, despite their ability to immunoprecipitate WNV NS1 (10). Similar to neutralization test results, functional antibodies detected by the CDC assay seem to be more virus specific than antibodies detected by a binding assay such as the ELISA.

The cutoff point for determining serum samples as positive for WNV NS1 antibodies is important for differentiating WNV from JEV infections in the present CDC assay. When 1:10 dilutions of sera were used, 5 of the 100 WNV NS1 antibody-negative sera showed positive results (Fig. 2). Further dilutions of sera decreased the number of false-positive samples to one at 1:20 and zero at 1:40 and 1:80 dilutions. Thus, the 1:40 or 1:80 dilution used in the one-dilution method with a cutoff for specific lysis of 19.8%, or in the end point method as a borderline dilution, is considered to provide stringent criteria for the CDC assay, with false-positive results unlikely to occur.

In conclusion, the present study showed that the CDC assay can measure WNV NS1 antibodies in horse sera. Thus, this assay would be a useful tool for differentiating WNV from JEV infections in serological diagnoses.

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Short Communication

Antibodies to Bovine Serum Albumin in Human Sera: Problems and Solutions with Casein-Based ELISA in the Detection of Natural Japanese Encephalitis Virus Infections

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SUMMARY: An ELISA system for measuring antibodies to nonstructural protein 1 (NS1) of Japanese encephalitis virus has already been established. This system uses an ELISA diluent containing casein, instead of bovine serum albumin (BSA). During a survey, we found that 21 (21%) of 102 children aged 1-5 years, who had no history of Japanese encephalitis vaccination and were without detectable neutralizing antibodies, showed positive results with this ELISA system. Western blotting analysis showed that sera from 19 (91%) of these 21 subjects had antibodies to BSA, but not NS1. These sera reacted with BSA antigen remaining in immunoaffinity-purified NS1 antigen. One solution to this problem was to reduce the BSA level to $\leq 1\%$ of the NS1 amount. Another was to use a control well sensitized with BSA with the same amount as that contained in the NS1 antigen preparation.

Japanese encephalitis virus (JEV) of the genus *Flavivirus* in the family *Flaviviridae*, is the leading cause of viral encephalitis in Asia (1). While Japanese encephalitis (JE) is vaccine preventable (2), the recommendation for JE vaccination in Japan was suspended in 2005 (3,4). The significance of a vaccination program is a topic that has generated much debate. This is because the current low JE incidence may also be attributable to the decreased number of vector mosquitoes along with the relocation of pig farms further away from residential areas. However, the natural infection rate in humans is a critical factor for evaluating any need for continuous vaccination.

Antibodies to JEV nonstructural protein 1 (NS1) constitute a marker of natural infection among populations vaccinated with an inactivated JE vaccine (5). Although ELISA is a serological method suited for antibody surveys, nonspecific reactions occurring in human sera have hampered the reliable measurement of low-level NS1 antibodies induced in subclinically infected humans. We have found that the nonspecific reactions occurring in a system using an ELISA diluent containing bovine serum albumin (BSA) were minimized when casein-based diluent was used (6).

The ELISA protocol has been described (6). The only characteristic feature compared with those of a conventional ELISA scheme was the ELISA diluent, which was composed of 0.05 M Tris-HCl (pH 8.0) containing 0.2% casein, 0.05% Tween 20, 1 mM EDTA, and 0.15 M NaCl. Briefly, microplates sensitized with purified NS1 antigens at 10 ng/well were incubated serially with test sera and alkaline phosphatase-conjugated goat anti-human IgG. Both were prepared in the ELISA diluent. The NS1 antigen used for sensitization was immunoaffinity-purified from culture fluids of NS1-expressing cells using a monoclonal antibody specific for NS1. Nonsensitized control wells were run in parallel and differences in absorbance from antigen-sensitized wells were regarded as NS1-specific reactions.

During an NS1 antibody survey using sera collected in Kumamoto Prefecture from 2004 to 2008 (7), we noticed that a large population (21%, 21 of 102) of children aged 1-5 years, who had no vaccination history and no detectable neutralizing antibodies, showed positive results in our ELISA for measuring NS1 antibodies. Although NS1 antibodies detected by our ELISA do not necessarily correlate with E antibodies detected by the neutralization test, we could find no specific reasons to explain this high prevalence of NS1 antibodies.

These 21 samples were then analyzed on Western blots. Since the antigen lot used for the ELISA that produced the above results (positive for NS1 antibodies) had run out by the time of the present study, a different lot was used as the antigen for this analysis. Of these, 19 (91%) showed a visible band at the molecular weight equivalent to that of BSA but did not show any bands corresponding to NS1. Three examples are shown in Fig. 1A. To confirm the reaction with BSA, we examined these samples for reactivity against varying amounts of BSA sensitized on an ELISA plate (Fig. 1B). The reaction was found to depend on the amount of BSA. These results indicated that some sera contained antibodies to BSA that may have caused false-positive results in the ELISA measuring NS1 antibodies. BSA antibodies and their effects on serological tests have

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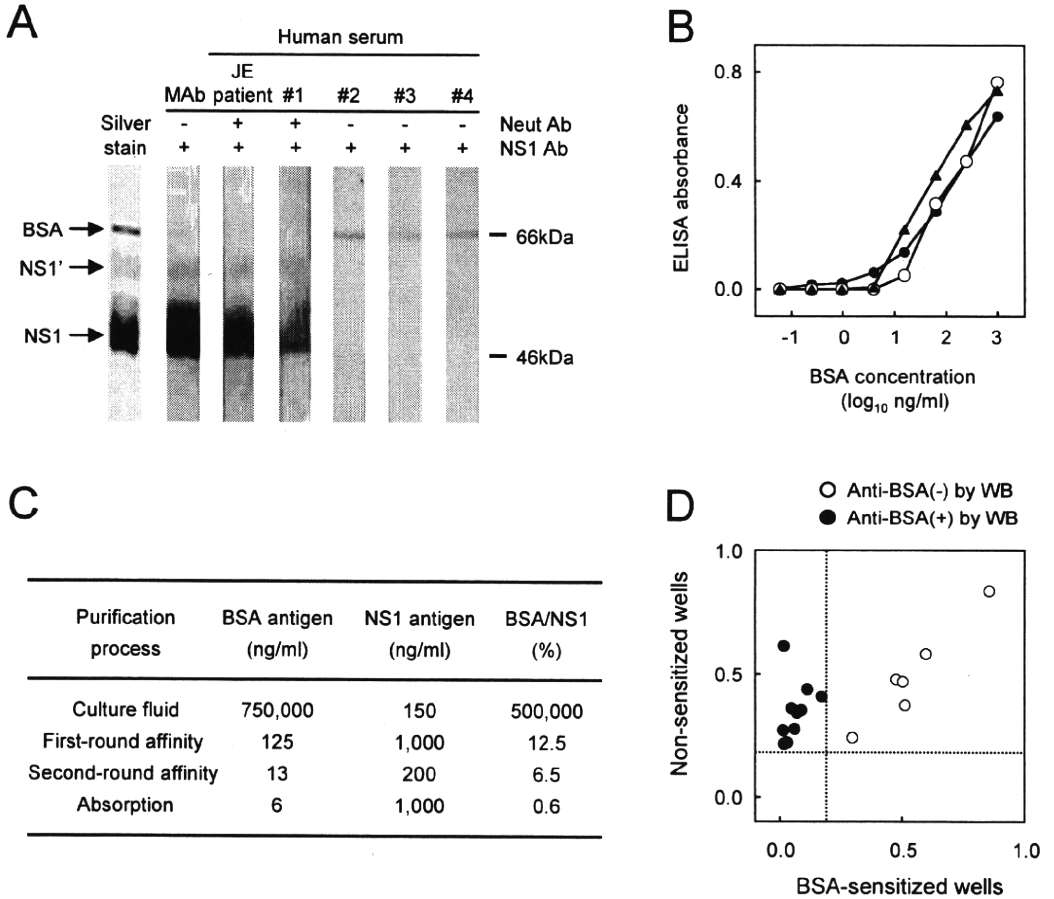

Purification process	BSA antigen (ng/ml)	NS1 antigen (ng/ml)	BSA/NS1 (%)
Culture fluid	750,000	150	500,000
First-round affinity	125	1,000	12.5
Second-round affinity	13	200	6.5
Absorption	6	1,000	0.6

Fig. 1. Reaction between BSA antibodies in human sera and BSA antigen contained in a purified NS1 antigen preparation. (A) Western blotting analyses of sera of children with no history of JE vaccination and with no detectable neutralizing antibodies. Analyses were performed essentially as previously described (6). Briefly, an immunoaffinity-purified NS1 antigen was run on standard Laemmli gels under nonreducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with test specimens and alkaline phosphatase-conjugated anti-human IgG. Test sera were collected in Kumamoto Prefecture (7): sera #2 (3-year-old female), #3 (4-year-old female), and #4 (5-year-old female) determined as negative (−) for neutralizing antibodies (Neut Ab) and positive (+) for NS1 antibodies (NS1 Ab) in our ELISA. As a reference, gels were silver-stained or blots were reacted with mouse monoclonal JE-6H4 (MAb) or sera from a JE patient and a subclinically infected human (#1). (B) Dose-dependent ELISA reactivities of human sera against varying concentrations of BSA used for sensitization of the plate. Samples were sera #2 (open circle), #3 (closed circle), and #4 (closed triangle). (C) Purification process and NS1 antigen purity. The antigen concentration of immunoaffinity purification was expressed as an average of the first four eluates. The "Second-round affinity" indicates that the eluates obtained from the "First-round affinity" process were repeatedly affinity-purified by the same procedure. "Absorption" indicates that the "First-round affinity" sample was incubated with rabbit anti-BSA coupled to Sepharose 4B beads at 0°C for 2 h. The latter two procedures were performed with a portion of the "First-round affinity" sample; with the antigen amounts obtained adjusted to those obtained after the "First-round affinity" process. (D) Effect of the use of BSA-sensitized wells on NS1 antibody levels. Using sera with (10 samples; closed circles) or without (6 samples; open circles) a band corresponding to BSA on Western blots, NS1 antibody levels obtained using BSA-sensitized wells were compared with the original NS1 antibody levels obtained using nonsensitized wells.

been reported in healthy human sera, particularly from infants, and are probably induced by dietary proteins (8,9).

The above analysis also showed the presence of BSA in this lot of the affinity-purified NS1 preparation. A direct ELISA using horseradish peroxidase-conjugated rabbit anti-BSA (Alpha Diagnostic International, San Antonio, Tex., USA) measured 1.25 ng of BSA in 10 ng of this NS1 antigen lot, corresponding to one plate well. It appeared that the immunoaffinity-purification strategy cannot always eliminate the BSA originally contained in the maintenance medium of NS1-expressing cells at a concentration of 0.075% (750 μg/ml). Therefore, minimizing the amount of BSA remaining in the NS1 antigen preparation appears a solution to the problem occurring in casein-based ELISA. The above dose-response curve (Fig. 1B) showed that the reaction was undetectable in an amount of BSA of $\leq$1 ng/ml ($\leq$0.1 ng/well), indicating that the effect of BSA on the present ELISA was minimized at $\leq$1% of the NS1 antigen (10 ng/well).

Attempts to minimize the relative amount of BSA by repeating immunoaffinity purification steps failed: a high BSA:NS1 ratio remained and there was a considerable loss of NS1 antigen (Fig. 1C). The most effective method to reduce the amount of BSA antigen was absorption with rabbit anti-BSA (Alpha Diagnostic Inter-

national) coupled to Sepharose 4B beads (NHS-activated Sepharose 4B Fast Flow; GE Healthcare UK, Buckinghamshire, England). Specifically, the absorption reduced the amount of BSA to $\leq 1\%$ of NS1 without loss of the NS1 antigen (Fig. 1C).

Another way to resolve the issue was the use of an equivalent amount of BSA in a nonsensitized control. Subtraction of the absorbances obtained with nonsensitized control wells from those obtained with antigen-sensitized wells is considered in theory, and is often used to reduce nonspecific reactions occurring in ELISA. Thus, we sensitized wells with BSA with the same amount contained in the purified NS1 antigen preparation (1.25 ng per well) and compared the results with those obtained using truly nonsensitized wells prepared by incubation only with ELISA coating buffer. As shown in Fig. 1D, 10 sera that were positive for BSA antibodies on Western blots turned negative for NS1 antibodies in ELISA when BSA-sensitized wells were used, whereas six NS1-positive sera that were negative for BSA antibodies on Western blots remained positive for NS1 antibodies.

In conclusion, the present study showed that BSA antibodies, which were found in a relatively large population of children, were the cause of false-positive results in our ELISA using casein-based diluent. However, the present study was able to minimize the effect of BSA antibodies by reducing the BSA level to $\leq 1\%$ in the NS1 antigen preparation, and achieved the same by using an equivalent amount of BSA in nonsensitized control wells. Although the use of medium without BSA would be a theoretical solution, some serum samples that were originally negative became positive when NS1 antigens prepared using serum-free or casein-containing medium were used.

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Evaluation of Extracellular Subviral Particles of Dengue Virus Type 2 and Japanese Encephalitis Virus Produced by *Spodoptera frugiperda* Cells for Use as Vaccine and Diagnostic Antigens[▽]

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New or improved vaccines against dengue virus types 1 to 4 (DENV1 to DENV4) and Japanese encephalitis virus (JEV), the causative agents of dengue fever and Japanese encephalitis (JE), respectively, are urgently required. The use of noninfectious subviral extracellular particles (EPs) is an inexpensive and safe strategy for the production of protein-based flavivirus vaccines. Although coexpression of premembrane (prM) and envelope (E) proteins has been demonstrated to produce EPs in mammalian cells, low yields have hindered their commercial application. Therefore, we used an insect cell expression system with *Spodoptera frugiperda*-derived Sf9 cells to investigate high-level production of DENV2 and JEV EPs. Sf9 cells transfected with the *prM* and *E* genes of DENV2 or JEV secreted corresponding viral antigens in a particulate form that were biochemically and biophysically equivalent to the authentic antigens obtained from infected C6/36 mosquito cells. Additionally, equivalent neutralizing antibody titers were induced in mice immunized either with EPs produced by transfected Sf9 cells or with EPs produced by transfected mammalian cells, in the context of coimmunization with a DNA vaccine that expresses EPs. Furthermore, the results of an enzyme-linked immunosorbent assay (ELISA) using an EP antigen derived from Sf9 cells correlated significantly with the results obtained by a neutralization test and an ELISA using an EP antigen derived from mammalian cells. Finally, Sf9 cells could produce 10- to 100-fold larger amounts of E antigen than mammalian cells. These results indicate the potential of Sf9 cells for high-level production of flavivirus protein vaccines and diagnostic antigens.

Dengue virus types 1 to 4 (DENV1-4) and Japanese encephalitis virus (JEV), the causative agents of dengue fever and Japanese encephalitis (JE), respectively, are globally important human pathogens (10) for which new or improved vaccines are urgently required. DENV1-4 cause dengue fever and dengue hemorrhagic fever in tropical areas and many subtropical areas. An estimated 50 million to 100 million dengue cases occur annually, with 2.5 billion people at risk of infection (11). However, there is no approved vaccine for dengue diseases, and the development of such a vaccine is urgently needed (12). JEV is the single largest cause of childhood viral encephalitis in the world, with an estimated 50,000 cases annually. Mortality rates can reach 30% among confirmed cases, and as many as one-third of survivors suffer from permanent and severe psychoneurological sequelae (13, 39). Although inactivated vaccines are used internationally for JE, they are too expensive for widespread use in most developing countries (3), and therefore, more cost-effective alternatives are needed.

Neutralizing antibodies are important in host protection against dengue diseases and JE (10, 34). For JE, previously used mouse brain-derived (16, 44) and more recently used Vero cell-derived (20, 25, 35) inactivated vaccines can efficiently induce neutralizing antibody responses. However, these protein-based vaccines are produced from infectious agents, and their production therefore requires biosafety level 2 or 3 containment facilities and complex purification protocols, thus increasing the cost of the vaccine. Vaccine production without infective procedures can be achieved using genetic engineering techniques (29, 55).

DENV1-4 and JEV are members of the genus *Flavivirus* in the family *Flaviviridae* (37). The envelope (E) protein is the major component of the envelopes of flavivirus virion particles and possesses most of the neutralizing epitopes (46). The other protein on the envelopes of mature virions is the membrane (M) protein, which is synthesized as the precursor membrane (prM) protein in infected cells. Cells expressing flavivirus prM and E proteins are known to secrete nucleocapsid-free subviral extracellular particles (EPs), which are similar to slowly sedimenting hemagglutinin (SHA) particles secreted from flavivirus-infected cells (47). EPs of JEV synthesized in mammalian expression systems have been evaluated for their immunogenicity and/or protective efficacy in mice (15, 24, 43). Two of these studies (24, 43) demonstrated that the EPs induced neutralizing antibodies at levels comparable to those induced by an inactivated JE vaccine. In our laboratory, mammalian cell lines continuously expressing EPs of dengue type 2 virus (DENV2) (26) or JEV (27) have been generated and designated D cells and F cells, respectively. The EPs contained an E protein that was antigenically and biochemically equivalent to the authentic E protein, and the EPs were immunogenic and protective in mice. However, the yields of viral antigens produced from D and F cells were low and would not meet the requirements for

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commercial vaccine production. Increasing the levels of viral antigen production from transfected cells would reduce the cost of vaccine preparation.

Recently, insect cell expression systems have been increasingly used in various fields of medical sciences (1, 6, 17, 53). In general, insect cells are easier to cultivate than mammalian cells, because they often do not require serum supplementation in the culture medium or incubation under CO_2. In addition, insect cells can be adapted to suspension culture, allowing cultures to be simply scaled up. Furthermore, various techniques have been developed for high-density culture of insect cells; for example, in one study, the immobilization of insect cells within biomass support particles achieved a density of approximately 3×10^7 cells/cm^3 (50). Thus, the insect cell expression system can be a simple and inexpensive strategy for vaccine antigen production.

In addition to their use as vaccine antigens, EPs derived from mammalian cells could be used as serodiagnostic antigens (27, 32). The production of serodiagnostic antigens may also encounter problems when the antigens are sourced directly from infectious agents. Currently, numerous commercial assays utilizing several different formats, such as the immunochromatography test and the IgM capture enzyme-linked immunosorbent assay (ELISA), are available for the diagnosis of DENV and JEV infections (4, 49). These commercial tests use viral antigens derived from transfected or infected cultured cells. Thus, the application of insect cell-derived EPs as diagnostic antigens would be an attractive alternative.

In this study, we produced EPs of DENV2 and JEV in a transient expression system using the Sf9 cell line, which was derived from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda*. These proteins were evaluated for vaccine and diagnostic antigens, mainly by direct comparison with mammalian-cell-derived EPs. The EPs produced from Sf9 cells were immunogenic in mice and useful as antigens for ELISA. In addition, Sf9 cells produced larger amounts of antigen than CHO cells, suggesting the potential applicability of insect cells for the production of DENV2 and JEV antigens for vaccines and serodiagnostic tests.

MATERIALS AND METHODS

Cells. Sf9 cells were purchased from Novagen (Darmstadt, Germany) and cultivated in BacVector-insect cell medium (Novagen). A mosquito cell line, C6/36, and mammalian cell lines, Vero and CHO-K1, have been described previously (27). Briefly, the growth medium was Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (60 μg/ml); for C6/36 and CHO-K1 cells, nonessential amino acids were also added. D and F cells, which are CHO-K1 cells genetically modified to continuously express EPs of DENV2 or JEV, respectively (26, 27), were grown in the same medium as CHO-K1 cells. Cells were cultivated at 28°C for insect cells or at 37°C for mammalian cells under a humidified atmosphere of 5% CO_2. The maintenance medium used for mammalian and C6/36 cells following transfection or infection was the growth medium containing 0.1% bovine serum albumin (BSA) in place of 10% FBS.

Viruses. The New Guinea C (NGC) strain of DENV2 (26) and the Nakayama strain of JEV (41) were used. Culture fluids harvested from C6/36 cells infected with each of these viruses were used for biochemical characterizations and neutralization tests. The virulent Beijing P3 strain of JEV, in the form of a 10% suckling mouse brain homogenate, was used for mouse challenge experiments (41).

Plasmids. The pcDNA3-based plasmids encoding the prM and E proteins of DEN2V strain NGC (designated pcD2ME [26]) or JEV strain Nakayama (designated pcJEME [27]) have been described previously. To construct appropriate expression plasmids for the insect cells, the *prM* and *E* genes were excised from the pcD2ME plasmid and were inserted into the pIB/V5His vector (Invitrogen, San Diego, CA) using the EcoRV and XhoI sites; this construct was designated pIBD2ME. Similarly, the *prM* and *E* genes were excised from the pcJEME plasmid and were inserted into the pIB/V5His vector using the EcoRI and XhoI sites; this construct was designated pIBJEME. Plasmid inserts were confirmed by sequencing. All plasmid DNAs (pcDNA3, pcD2ME, pcJEME, pIBD2ME, and pIBJEME) were purified using a Qiagen plasmid kit (Qiagen, Hilden, Germany).

Antibodies. Rabbit polyclonal antibodies to DENV2 (26) or JEV (21) have been described previously. Briefly, these were obtained by repeated immunization of a rabbit with a virion fraction that was dissociated with 0.05% Triton X-100. Mouse monoclonal antibodies reactive with JEV antigens were JE-10B4 (specific for JEV E) (30) and J2-2F1 (specific for JEV prM/M) (40) (provided by Mary K. Gentry of the Walter Reed Army Institute of Research, Washington, DC), while those reactive with dengue virus antigens were D3-2H2 (specific for prM) (14), D2-3H5 (specific for E) (9), and D1-4G2 (specific for E) (14) (obtained from hybridomas purchased from the American Type Culture Collection, Manassas, VA).

Sera. The serum samples used for evaluating EPs as ELISA antigens were those previously obtained from mice immunized with pcD2ME or pcJEME (28).

Transfection. Sf9 cells were transfected with 1 to 4 μg of pIBD2ME or pIBJEME by using the FuGENE HD transfection reagent (Roche, Mannheim, Germany), according to the manufacturer's instructions. Similarly, CHO-K1 cells were transfected with 1 to 4 μg of pcD2ME or pcJEME using the same transfection reagent. For investigation of the yield, these cells were then incubated in growth medium. For the preparation of immunogens, transfected cells were incubated in the maintenance medium.

Immunochemical staining. Immunochemical staining was performed essentially as described previously (27). Briefly, cells were fixed with a mixture of cold methanol and acetone (1:1) and were then dried. These cells were then incubated serially with monoclonal antibodies to JEV or DENV2, biotinylated anti-mouse IgG, ABC (avidin-biotinylated peroxidase complex) reagents, and the VIP substrate (Vector Laboratories, Inc., Burlingame, CA).

Sedimentation analysis. Samples were applied to a 10 to 40% (wt/wt) or a 10 to 50% continuous sucrose density gradient prepared in TN buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl). Following centrifugation at 55,000 rpm for 90 min at 4°C in the S55S rotor of a Himac CS100GX micro-ultracentrifuge (Hitachi Koki, Ibaraki, Japan), fractions were collected from the bottom of the tube. Each fraction was tested for the level of E antigen by ELISA (see below) and for infectivity by using a plaque assay on Vero cell monolayers.

Purification of extracellular particles. EPs were purified based on a method described previously (27). Briefly, culture supernatants harvested from transfected cells were clarified and precipitated with 10% polyethylene glycol (PEG; molecular mass, approximately 6,000 Da). Following centrifugation, the pellets were suspended in TN buffer and were applied to a sucrose density gradient (see above). The collected fractions were examined for E antigen levels by ELISA (see below). The fractions containing the highest and the second highest levels of E antigen were used as purified EPs. EPs obtained from Sf9 cells transfected with pIBD2ME or pIBJEME were designated D2EP-Sf9 or JEEP-Sf9, respectively. Similarly, those obtained from CHO-K1 cells transfected with pcD2ME or pcJEME were designated D2EP-CHO or JEEP-CHO, respectively. Those obtained with D cells and F cells were designated D2EP-D and JEEP-F, respectively.

ELISA for quantification of E antigen. The DENV2 and JEV E antigens were quantified by a sandwich ELISA as previously described (27). Briefly, 96-well microplates sensitized with a rabbit polyclonal antibody were serially incubated with samples at a dilution of 1:10 (for sucrose gradient fractions) or at serial 2-fold dilutions (for culture fluid samples), a monoclonal anti-E antibody (D2-3H5 or JE-10B4), alkaline phosphatase-conjugated anti-mouse IgG, and *p*-nitrophenyl phosphate. Antigen levels were calculated from the absorbance values obtained with the sample and a reference standard. The reference standard was prepared with D2EP-D or JEEP-F fractions, and the amount of E protein contained in the reference standard was estimated by comparison of an E preparation concentrated from the reference standard with the BSA samples on silver-stained gels.

Endoglycosidase treatment. *N*-Glycosidase F (PNGase F; Roche Diagnostics, Basel, Switzerland) was used according to the manufacturer's instructions, with some modifications. Purified EPs were boiled for 5 min with sodium dodecyl sulfate (SDS) at a final concentration of 1%. After cooling, the sample was divided into two equal aliquots and was mixed with the "reaction" buffer to make final concentrations of 64 mM phosphate buffer (pH 6.4), 50 mM EDTA, and 1% Triton X-100. One unit of PNGase F was then added to one of the two aliquots.