Virology **268,** 49–55 (2000)
doi:10.1006/viro.1999.0142, available online at http://www.idealibrary.com on IDEAL®

Japanese Encephalitis DNA Vaccine Candidates Expressing Premembrane and Envelope Genes Induce Virus-Specific Memory B Cells and Long-Lasting Antibodies in Swine

Eiji Konishi,*[,2] Masaoki Yamaoka,*[,1] Ichiro Kurane,† and Peter W. Mason‡

*Department of Health Sciences, Kobe University School of Medicine, Kobe 654-0142, Japan; †Department of Virology 1, National Institute of Infectious Diseases, Tokyo 162-8640, Japan; and ‡Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, New York 11944

Received July 26, 1999; returned to author for revision November 29, 1999; accepted December 14, 1999

Swine are an important amplifier of Japanese encephalitis (JE) virus in the paradomestic environment. In this study, two JE DNA vaccine candidates were evaluated for immunogenicity in swine. Both vaccine plasmids encode a cassette consisting of the signal of premembrane (prM), prM, and envelope (E) coding regions of JE virus. One plasmid, designated pcJEME, is based on a commercial vector (pcDNA3), whereas the other plasmid, designated pNJEME, is based on a vector (pNGVL4a) designed to address some of the safety concerns of DNA vaccine use. No differences were detected in the immunogenicity of these two plasmids in mice or swine. Swine immunized with the DNA vaccines at a dose of 100 to 450 μg at an interval of 3 weeks developed neutralizing and hemagglutination-inhibitory (HAI) antibody titers of 1:40 to 1:160 at 1 week after the second immunization. However, swine administered two doses of a commercial JE vaccine (formalin-inactivated virus preparation; JEVAX-A) developed low (1:10) or undetectable antibody responses after their boost. Interestingly, serum antibody titers elicited by DNA vaccines in swine were higher than those detected in mice. Eight days after boosting with viral antigen (JEVAX-A) to detect an anamnestic response, swine immunized two times with the DNA vaccine showed a >100-fold elevation in HAI titer, indicating a strong recall of antibody response. Swine maintained detectable levels of HAI antibody for at least 245 days after two immunizations with a DNA vaccine. These results indicate that these DNA vaccines are able to induce virus-specific memory B cells and long-lasting antibodies in swine, which were of higher levels than those obtained with a commercial formalin-inactivated JE vaccine.

INTRODUCTION

Japanese encephalitis (JE) virus, which belongs to the family Flaviviridae, causes infection of the central nervous system in humans and equines and stillbirths in swine (Shope, 1980; Monath, 1986). The virus has a zoonotic transmission cycle between birds and mosquitoes, with swine serving as an intermediate amplifier from which anthrophilic mosquitoes become infected, spreading virus to humans (Scherer *et al.,* 1959; Konno *et al.,* 1966; Oya, 1967). Therefore, mass vaccination of swine can prevent disease in swine and help to prevent JE epidemics in humans (Igarashi, 1992). Current JE vaccines for use in swine consist of attenuated or inactivated virus (Fujisaki, 1975; Inoue, 1975; Kurata, 1980a, b; Yoshida, 1981; Yamagishi, 1989), and there are concerns about the safety and cost of producing and using these products. Recently, molecular biology has been used to develop experimental vaccines for several viral diseases, which could overcome some of the drawbacks of existing vaccines. DNA vaccines are one promising approach, because they are based on the delivery of inexpensive product that encodes only a portion of a viral genome, enhancing the cost and safety over traditional vaccines (Manickan *et al.,* 1997).

For the development of second-generation vaccines against flavivirus diseases, we used a cassette encoding the signal sequence of premembrane (prM), prM, and full-length E proteins. Poxviruses that deliver the signal-prM-E cassette induce expression of subviral extracellular particles (containing prM/M and E) in cells and elicit neutralizing antibody and protective immunity in mice (Mason *et al.,* 1991; Konishi *et al.,* 1991, 1992, 1994). The use of the signal-prM-E cassette to induce production of subviral particles and/or neutralizing antibody in mice has been reported for a different strain of JE virus (Sato *et al.,* 1993) and other flaviviruses, such as yellow fever (Pincus *et al.,* 1992), West Nile (Yamshchikov and Compans, 1993), dengue type 1 (Fonseca *et al.,* 1994) and tick-borne encephalitis (Allison *et al.,* 1995) viruses. In addition to their immunogenicity in mice, poxviruses encoding the JE virus signal-prM-E gene cassette have been reported to elicit neutralizing antibody in swine and humans (Konishi *et al.,* 1992, 1998a). Furthermore, we have demonstrated that memory cytotoxic T lymphocytes (CTLs), another important arm of immunity, are induced

[1] Present address: Hyogo Prefectural Institute of Public Health, Kobe 652-0032, Japan.
[2] To whom reprint requests should be addressed at 7-10-2 Tomogaoka, Suma-ku. Fax: +81-78-796-4594. E-mail: ekon@ams.kobe-u.ac.jp.

0042-6822/00 $35.00

in mice and humans by this gene cassette (Konishi *et al.,* 1997, 1998a). We developed a pcDNA3-based DNA vaccine containing the signal-prM-E gene cassette of JE virus (designated pcJEME here; it is identical to pcDNA3JEME reported in Konishi *et al.,* 1998b), and demonstrated an ability of pcJEME to induce neutralizing antibody, memory CTLs, and protective immunity in mice (Konishi *et al.,* 1998b). The induction of protective immunity against flaviviruses by other DNA vaccines has been reported using the nonstructural (NS) 1 gene of JE virus (Lin *et al.,* 1998), the signal, prM and truncated E genes of dengue type 2 virus (Kochel *et al.,* 1997; Porter *et al.,* 1998), and the signal, prM, and full-length E genes of St. Louis (Phillpotts *et al.,* 1996) and tick-borne (Schmaljohn *et al.,* 1997) encephalitis viruses.

Although pcJEME displayed promising properties in murine studies, the vector used to construct this vaccine candidate contains an ampicillin-resistance gene and sequences derived from SV40 virus. The former element is thought to be undesirable because it could be incorporated into pathogenic bacteria in inoculated animals, rendering them resistant to useful antibiotics, whereas the latter element is thought to be potentially dangerous because SV40 is an oncogenic virus that transforms human cells in tissue culture (Noonan and Butel, 1978). Therefore, we selected an alternative vector, pNGVL4a, which uses a kanamycin-resistance gene and does not contain any SV40-derived sequences (Hartikka *et al.,* 1996; Doolan *et al.,* 1997), to construct a new prM/E-encoding DNA vaccine candidate, pNJEME.

The purpose of the present study was to evaluate immunogenicity of pcJEME and pNJEME in swine. Both of the JE DNA vaccine candidates induced neutralizing and hemagglutination-inhibiting (HAI) antibodies in swine at levels greater than those detected in inactivated vaccine-vaccinated animals. Furthermore, DNA-vaccinated swine showed a brisk recall immune response and long-lived antibody.

RESULTS

In vitro expression of vaccine plasmids

pcJEME and pNJEME specify the expression of prM and E *in vitro.* CHO-K1 cells transfected with 0.5 μg of pcJEME or pNJEME were immunostained with monoclonal antibodies to prM (J2-2F1; provided by Dr. Mary K. Gentry of Walter Reed Army Institute of Research, Washington, DC) or E (J3-11B9; Mason *et al.,* 1989) to determine the levels of viral antigens specified by these plasmids. Interestingly, these studies failed to detect any difference in the levels of antigen produced by these two plasmids, even though pNJEME contains an intron, which was expected to improve its ability to produce antigen-encoding mRNA.

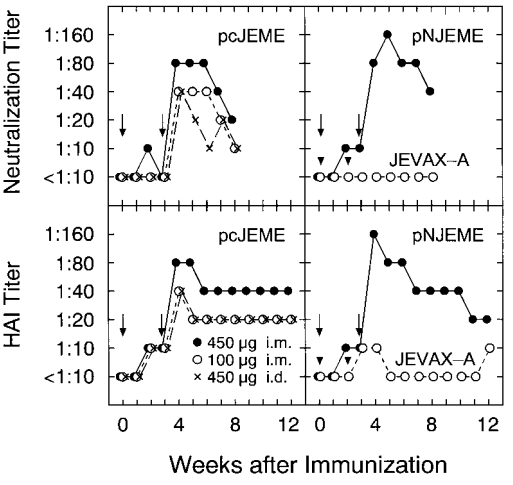

FIG. 1. Time course of neutralizing (top) and HAI (bottom) antibody titers determined for sera pooled from each group of five immunized pigs. (Left) Pigs were immunized i.m. or i.d. with pcJEME at a dose of 450 or 100 μg. (Right) Pigs were immunized i.m. with pNJEME at a dose of 450 μg or s.c. with JEVAX-A at the single dose recommended by the manufacturer. Immunizations were performed at weeks 0 and 3 for pcJEME and pNJEME (arrows) and at weeks 0 and 2 for JEVAX-A (arrowheads). Pigs were bled 16 days before the first immunization (week 0) and every week until week 12. Neutralization titers were obtained with sera collected until week 8.

Immunogenicity of pNJEME in mice

Mice immunized with pNJEME developed a neutralizing antibody titer of 1:10 at 2 weeks after the second immunization. In controls, mice immunized with pcJEME or a commercial JE vaccine for human use (JEVAX-H) developed an equivalent titer (1:10), whereas no detectable neutralizing antibody was obtained in mice immunized with pNGVL4a. After challenge with a lethal dose of the P3 strain of JE virus, five of six mice immunized with pNJEME survived for 21 days. The same survival rate was obtained with mice immunized with pcJEME or the JEVAX-H, whereas all mice inoculated with pNGVL4a died of challenge. These results indicate that immunization with pNJEME induces neutralizing antibody and protective immunity in mice. The induction of neutralizing antibody and protective immunity by pcJEME has been reported previously (Konishi *et al.,* 1998b).

Induction of neutralizing and HAI antibodies in swine

Five groups of five pigs were inoculated twice with pNJEME, pcJEME, or a commercial JE vaccine for animal use (JEVAX-A) and bled every week until week 12 after the first inoculation. Serum levels of neutralizing and HAI antibody titers were assessed until weeks 8 and 12, respectively. The time courses of neutralizing and HAI antibodies in pooled sera are shown in Fig. 1, and the distributions of individual antibody titers on weeks 4 and 7 are shown in Fig. 2. Variations of individual antibody titers were 2- to 8-fold among each group and were

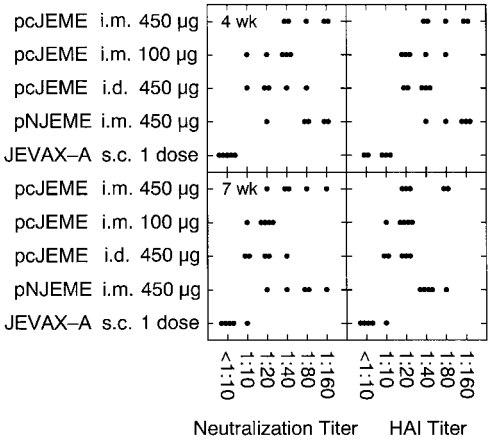

FIG. 2. Neutralizing (left) and HAI (right) antibody titers observed in sera from individual pigs on week 4 (top) and week 7 (bottom) after the first immunization. Groups of five pigs were immunized i.m. or i.d. with 450 or 100 μg of pcJEME, i.m. with 450 μg of pNJEME, or s.c. with one dose of JEVAX-A (see legend to Fig. 1 for details).

observed for both neutralizing and HAI antibodies (Fig. 2). No systemic or local reactions were observed in any swine after intramuscular (i.m.) or intradermal (i.d.) inoculation with plasmid DNAs. As expected from earlier studies (Konishi *et al.,* 1992), HAI and neutralizing antibody titers were directly related to each other for each individual animal and for each group of swine.

Swine immunized by i.m. inoculation with 450 μg of pcJEME developed a neutralization titer of 1:10 at 2 weeks after the first immunization and a titer of 1:80 at 1 week after the second immunization (Fig. 1). The neutralization titer was maintained at 1:80 for 2 weeks and then gradually declined. In the 100-μg inoculation group, detectable levels of neutralizing antibodies were not observed before the second immunization, with a neutralization titer of 1:40 detected 1 week after the second immunization. As expected (see earlier), the time course of HAI antibody titers (Fig. 1) and individual antibody titers on weeks 4 and 7 (Fig. 2) showed the same relationship. Taken together, these results indicate that pcJEME is able to induce neutralizing and HAI antibodies in all swine vaccinated with two doses of 100 and 450 μg and that the higher dose elicited higher titers of antibodies in both tests.

Examination of the immunogenicity of 450 μg of pcJEME by the i.d. route revealed a similar time course of antibody production (Fig. 1) and a consistent immune response among vaccinated animals (Fig. 2). However, the i.d. route elicited lower levels of antibodies (as measured by both neutralization and HAI assays) than i.m. inoculation (Figs. 1 and 2).

Swine inoculated i.m. with 450 μg of pNJEME developed neutralizing and HAI antibodies in a manner indistinguishable from swine inoculated i.m. with 450 μg of pcJEME (Figs. 1 and 2). Thus, with these criteria for immunogenicity, pNJEME was as effective as the ampicillin-resistance gene- and SV40 DNA-containing pcJEME plasmid as a DNA vaccine candidate.

Swine vaccinated twice with JEVAX-A developed HAI antibody titers at a maximum of 1:10 within the 12-week experimental period, but no detectable levels of neutralizing antibodies were observed in pooled sera for 8 weeks after the first immunization (Fig. 1). These results indicate that our DNA vaccines induce stronger immune responses in swine than a currently approved JE vaccine.

Anamnestic HAI antibody responses in pNJEME-immunized swine

To examine an ability of pNJEME to induce memory B and helper T cells, the swine immunized twice with pNJEME or JEVAX-A were boosted with JEVAX-A (three pigs from each group) or mock-boosted with saline (remaining two pigs in each group) and examined for the anamnestic responses with the HAI antibody test (Fig. 3). All three of the pNJEME-immunized pigs that were boosted had an HAI titer of 1:20 before boosting. After boosting, these swine developed HAI titers from 1:20 to 1:40 on day 2, from 1:40 to 1:80 on day 4, and 1:2560 on day 8 (Fig. 3). The three JEVAX-A-immunized pigs had HAI titers from <1:10 to 1:10 before boosting and from 1:10 to 1:80 on day 8. Mock-boosted swine had the same HAI titers on days 0 and 8 in both groups. Thus pNJEME-immunized swine produced a significantly stronger anamnestic antibody response than swine immunized with JEVAX-A, suggesting that pNJEME induces more memory B and helper T cells than the currently used commercial vaccine.

Duration of immunity induced by pNJEME in swine

Sera were periodically collected from two pigs immunized twice with pNJEME and two pigs immunized twice

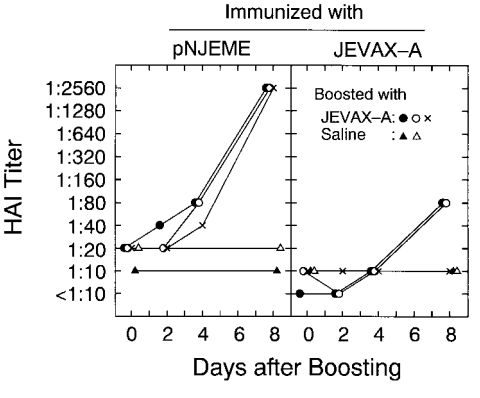

FIG. 3. Anamnestic HAI antibody responses to viral antigen in swine immunized twice with 450 μg of pNJEME (left) or twice with the manufacturer's recommended dose of JEVAX-A (right). Three of five pigs in each group were boosted s.c. with 2 ml of JEVAX-A on day 116. As a control, the remaining two pigs were mock-boosted s.c. with 2 ml of saline. Sera were collected before boosting (day 0) and 2, 4, and 8 days after boosting and examined in the HAI test.

TABLE 1

Duration of Immunity Induced by pNJEME in Swine

Day after the first immunization[b]	HAI titer			
	JEVAX-A[a]		pNJEME	
	No. 107[c]	No. 108	No. 127	No. 128
84	<1:10	<1:10	1:10	1:20
98	<1:10	<1:10	1:10	1:20
116	<1:10	<1:10	1:10	1:20
124	<1:10	<1:10	1:10	1:20
154	<1:10	<1:10	1:10	1:40
195	<1:10	<1:10	1:20	1:80
217	<1:10	<1:10	1:20	1:80
245	<1:10	<1:10	1:20	1:40

[a] Inactivated vaccine for animal use.

[b] Pigs were inoculated with one dose of JEVAX-A twice on days 0 and 14 or with 450 μg of pNJEME on days 0 and 21. All four pigs shown were injected with saline on day 116 as a mock-boosted control in the experiment to detect anamnestic responses (see Materials and Methods for details).

[c] Animal number.

with JEVAX-A until the end of the experimental period (day 245 after the first immunization) and were examined for HAI antibody levels (Table 1). The swine immunized with pNJEME showed detectable levels of HAI antibody from day 84 to day 245, and the HAI titers tended to increase with increasing period after the immunization. In contrast, no detectable levels of HAI antibodies were obtained in two pigs immunized with JEVAX-A in this period. This result indicates that immunization with pNJEME induces long-lasting antibody responses in swine.

DISCUSSION

Several devices to increase the levels of expression and/or immunogenicity of JE viral proteins were incorporated into pcJEME and pNJEME. Both vaccine candidates contain a strong eukaryotic promoter derived from human cytomegalovirus, a polyadenylation signal, a strong eukaryotic initiation site (an ACC sequence preceding the AUG start codon; Kozak, 1986), and the immunostimulatory sequences on the native (pcJEME) or modified (pNJEME) ampicillin-resistance gene (Sato *et al.,* 1996). In addition to these devices, pNJEME contained an intron A sequence downstream of the cytomegalovirus promoter and upstream of the eukaryotic initiation site. An addition of the intron A sequence has been reported to increase the level of gene expression (Chapman *et al.,* 1991). In the present study, however, pcJEME and pNJEME induced similar levels of both expression *in vitro* and immunogenicity in mice and swine. Explanations for the failure of the intron A to increase levels of expression include the possibility that mRNA processing is not the rate-limiting step in synthesis of the protein (which may include the assembly and release of the extracellular prM/E-containing particles). Alternatively, the products of the JE gene cassette could display some toxic properties, which could be exacerbated by enhanced synthesis.

Swine showed relatively high levels of antibody responses to DNA plasmids, although two immunizations were carried out on contralateral sides, which may result in weaker immune responses than ipsilateral inoculations. Furthermore, the DNA plasmids induced higher antibody levels in swine than in mice, which developed only low or undetectable levels of antibody as determined by the same serological methods in this and previous studies (Konishi *et al.,* 1998b). Specifically, two i.m. inoculations with 100 μg of pcJEME provided 4-fold higher neutralizing antibody titers in swine (1:80) than in mice (1:20 in sera pooled from five animals 2 weeks after the second immunization). This was in contrast to our previous data showing that the same JE virus prM/E gene cassette vectored by a vaccinia virus induced weaker humoral immune responses in swine than in mice (Konishi *et al.,* 1992). We did not investigate CTL responses in this study due to the difficulty of performing the assays on outbred animals and because our previous study indicated that CTLs induced by pcJEME are less important than neutralizing antibody in protecting mice from JE (Konishi *et al.,* 1999).

As an alternative to the live-virus challenge experiment, we boosted immunized swine with a viral antigen and examined anamnestic antibody responses to evaluate the potential protective capacity of pNJEME in swine. We previously demonstrated that low levels of neutralizing antibodies are able to protect swine from viremia and that strong anamnestic responses are elicited on challenge of swine (Konishi *et al.,* 1992). Furthermore, our previous study of postchallenge antibody responses in mice immunized with pcJEME demonstrated that neutralizing antibody produced after challenge is the critical component for protection of mice from lethal infection (Konishi *et al.,* 1999). Immunized mice that showed the significant elevation of neutralization titers on day 4 after challenge were protected, whereas none of the unimmunized mice showed any significant elevation and were not protected (Konishi *et al.,* 1999). The presence of memory B and helper T cells in mice inoculated with pcJEME appears to be important in postchallenge antibody responses and subsequent protection. In the present study, a rapid elevation of HAI antibody titers after boosting with JEVAX-A was observed in all three pigs immunized with pNJEME. Because the timing of elevation coincides well with the period of viremia in swine experimentally infected with JE virus (Kodama *et al.,* 1968; Fujisaki *et al.,* 1975; Sasaki *et al.,* 1982; Konishi *et al.,* 1992) and because HAI antibody titers correlated with neutralizing antibody titers in swine (Figs. 1 and 2),

it appears likely that two immunizations with pNJEME would prevent viremia in swine.

The commercially available inactivated JE vaccine JEVAX-A did not elicit high levels of antibodies in swine in the present study, although the immunization protocol was in accord with the instructions supplied by the manufacturer. However, the immunogenicity of JEVAX-A was demonstrated, because swine immunized with pNJEME developed extremely high levels of antibody responses after boosting with JEVAX-A. Two immunizations plus one-boost immunization with JEVAX-A, for a total of three inoculations, induced a modest level of antibody response. In conclusion, pcJEME and pNJEME induced higher levels of antibody responses than the commercial inactivated JE vaccine in swine. Also, the levels of antibody responses induced by these DNA vaccine plasmids were comparable or higher than those induced in swine by a recombinant vaccinia virus encoding the JE virus signal-prM-E gene cassette (vP923; Konishi *et al.,* 1992). Thus, pNJEME appears to be a promising JE DNA vaccine candidate for the prevention of viremia in swine and control of the transmission of JE virus infection to humans.

MATERIALS AND METHODS

Plasmids

The construction of pcJEME, which is a pcDNA3-based plasmid encoding the JE virus (Nakayama strain) signal sequence of prM, prM, and E genes, has been described previously (Konishi *et al.,* 1998b). The pNGVL4a vector, which possesses an eukaryotic promoter derived from human cytomegalovirus with the intron A sequence, a polyadenylation signal, and an immunostimulatory sequence on the modified ampicillin-resistance gene (Sato *et al.,* 1996), and lacking SV40-derived sequences, was supplied by the National Gene Vector Laboratory (University of Michigan). For construction of pNJEME, the JE virus signal-prM-E gene cassette was cloned from pcJEME into the pNGVL4a vector and resequenced. All plasmid DNAs (pcJEME, pcDNA3, pNJEME, pNGVL4a) were purified using a Qiagen Plasmid Kit (Funakoshi Co. Ltd., Tokyo, Japan).

Inactivated vaccines

The formalin-inactivated, mouse brain-derived JE vaccines for human use (JEVAX-H; Institute for Microbial Diseases of Osaka University, Kagawa, Japan) and for animal use (JEVAX-A; Institute of Chemical and Serum Therapy, Kumamoto, Japan) were selected in this study, because the Nakayama strain is used to prepare these vaccines.

Experiments in mice

Four groups of six 6-week-old male BALB/c mice were inoculated i.m. with 100 μg of pNJEME, pcJEME, or pNGVL4a or intraperitoneally (i.p.) with 1/10 dose of JEVAX-H twice at an interval of 2 weeks (at 6 and 8 weeks of age). Two weeks after the second immunization (at 10 weeks of age), these mice were bled retroorbitally for serological evaluation. One week after the bleeding (at 11 weeks of age), the six mice were challenged by i.p. injection with a dose of 50,000 LD_{50} of the Beijing P3 strain of JE virus (Mason *et al.,* 1991) and observed for 3 weeks.

Experiments in swine

Five groups of five 8- to 12-week-old pigs with a body weight of 13–14 kg (castrated males and females of the Yorkshire breed) were inoculated with 100 or 450 μg of pcJEME or 450 μg of pNJEME twice on days 0 and 21 or with the recommended dose of JEVAX-A on days 0 and 14. The plasmid DNAs diluted in PBS were injected i.m. at a single site on the neck (in the trapezius muscle) with a volume of 1 ml or i.d. at two sites on the base of the ear with a volume of 200 μl for each site. The JEVAX-A was inoculated subcutaneously (s.c.) with a volume of 1 ml on the neck. For injection of all immunogens, the right side of each animal was used for the first immunization and the left side was used for the second immunization. Swine were bled 16 days before the first immunization and every week for 12 weeks after the first immunization. Serum samples were isolated from blood, pooled, and examined for neutralizing and/or HAI antibodies. Individual sera collected on weeks 4 and 7 were also tested. All swine were monitored for adverse clinical manifestations due to vaccination. Anamnestic antibody responses were examined by boosting with a viral antigen on day 116 using two groups of five pigs immunized with pNJEME or JEVAX-A. Three of five pigs in each group were boosted by s.c. inoculation with 2 ml of JEVAX-A, and the remaining two for each group were mock-boosted s.c. with 2 ml of saline. Three boosted pigs were bled on day 116 (just before boosting) and on days 2, 4, and 8 after boosting (days 118, 120, and 124). Two mock-boosted pigs were bled on days 116 and 124. Sera from all of these samples were tested individually for HAI antibody. To examine the duration of antibody, sera were monitored periodically from the two pigs from each of pNJEME- or JEVAX-A-immunization groups, which were mock boosted with saline on day 116.

Serological tests

Neutralizing antibodies elicited in immunized mice or swine were determined using the Nakayama strain of JE virus as previously described (Konishi *et al.,* 1999). The neutralization titer was expressed as the greatest serum dilution yielding a 90% reduction in plaque number. The HAI test was performed as previously described (Mason *et al.,* 1991), except for the use of a commercial JE virus

antigen for diagnostic use (Takeda Pharmaceutical Co., Osaka, Japan).

ACKNOWLEDGMENTS

We thank Dr. Mikio Nakayama of the National Institute of Infectious Diseases for providing JEVAX-H and JEVAX-A, Dr. Mary K. Gentry of Walter Reed Army Institute of Research for providing monoclonal antibodies, and the National Gene Vector Laboratory (University of Michigan) for providing the pNGVL4a vector. The construction of pNGVL4a was supported by the grant from the National Institutes of Health (Grant #U42-RR-11149). The present study was supported in part by Research on Emerging and Re-emerging Infectious Diseases, the Ministry of Health and Welfare of Japan, and the Program for Sending Japanese Researchers to Foreign Institutes, Japan Health Sciences Foundation.

REFERENCES

Allison, S. L., Stadler, K., Mandl, C. W., Kunz, C., and Heinz, F. X. (1995). Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. *J. Virol.* **69,** 5816–5820.

Chapman, B. S., Thayer, R. M., Vincent, K. A., and Haigwood, N. L. (1991). Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res.* **19,** 3979–3986.

Doolan, D. L., Hedstrom, R. C., Wang, R., Sedegah, M., Scheller, L. F., Hobart, P., Noorman, J. A., and Hoffman, S. L. (1997). DNA vaccines for malaria: the past, the present, & the future. *Indian J. Med. Res.* **106,** 109–119.

Fonseca, B. A. L., Pincus, S., Shope, R. E., Paoletti, E., and Mason, P. W. (1994). Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice. *Vaccine* **12,** 279–285.

Fujisaki, Y., Sugimori, T., Morimoto, T., and Miura, Y. (1975). Development of an attenuated strain for Japanese encephalitis live virus vaccine for porcine use. *Natl. Inst. Anim. Health Q. Tokyo.* **15,** 15–23.

Hartikka, J., Sawdey, M., Cornefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M., Hobart, P., Norman, J., and Manthorpe, M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum. Gene. Ther.* **7,** 1205–1217.

Igarashi, A. (1992). Japanese encephalitis: Virus, infection, and control. *In* "Control of Virus Diseases" (E. Kurstak, Ed.), 2nd ed., pp. 309–342. Marcel Dekker, New York.

Inoue, Y. K. (1975). An attenuated Japanese encephalitis vaccine. *Prog. Med. Virol.* **19,** 247–256.

Kochel, T., Wu, S. J., Raviprakash, K., Hobart, P., Hoffman, S., Porter, K., and Hayes, C. (1997). Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. *Vaccine* **15,** 547–552.

Kodama, K., Sasaki, N., and Inoue, Y. K. (1968). Studies of live attenuated Japanese encephalitis vaccine in swine. *J. Immunol.* **100,** 194–200.

Konishi, E., Kurane, I., Mason, P. W., Shope, R. E., and Ennis, F. A. (1997). Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus-specific $CD8^+$ cytotoxic T lymphocytes in mice. *Virology* **227,** 353–360.

Konishi, E., Kurane, I., Mason, P. W., Shope, R. E., Kanesa-Thasan, N., Smucny, J. J., Hoke, C. H., Jr., and Ennis, F. A. (1998a). Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. *Vaccine* **16,** 842–849.

Konishi, E., Pincus, S., Fonseca, B. A. L., Shope, R. E., Paoletti, E., and Mason, P. W. (1991). Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* **185,** 401–410.

Konishi, E., Pincus, S., Paoletti, E., Laegreid, W. W., Shope, R. E., and Mason, P. W. (1992). A highly attenuated host-range restricted vaccinia virus strain, NYVAC, encoding the prM, E and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology* **190,** 454–458.

Konishi, E., Pincus, S., Paoletti, E., Shope, R. E., and Mason, P. W. (1994). Avipox virus-vectored Japanese encephalitis virus vaccines: use as vaccine candidates in combination with purified subunit immunogens. *Vaccine* **12,** 633–638.

Konishi, E., Yamaoka, M., Khin-Sane-Win, Kurane, I., and Mason, P. W. (1998b). Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding JE virus premembrane and envelope genes. *J. Virol.* **72,** 4925–4930.

Konishi, E., Yamaoka, M., Khin-Sane-Win, Kurane, I., Takada, K., and Mason, P. W. (1999). Anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. *J. Virol.* **73,** 5527–5534.

Konno, J., Endo, K., Agatsuma, H., and Ishida, N. (1966). Cyclic outbreaks of Japanese encephalitis among pigs and humans. *Am. J. Epidemiol.* **84,** 292–300.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44,** 283–292.

Kurata, K. (1980a). Outline of vaccines for animal use. IV. Japanese encephalitis live vaccine. *J. Vet. Med. Sci.* **33,** 85–87.

Kurata, K. (1980b). Outline of vaccines for animal use. V. Japanese encephalitis inactivated vaccine. *J. Vet. Med. Sci.* **33,** 132–134.

Lin, Y. L., Chen, L. K., Liao, C. L., Yeh, C. T., Ma, S. H., Chen, J. L., Huang, Y. L., Chen, S. S., and Chiang, H. Y. (1998). DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. *J. Virol.* **72,** 191–200.

Manickan, E., Karem, K. L., and Rouse, B. T. (1997). DNA vaccines—A modern gimmick or a boon to vaccinology? *Crit. Rev. Immunol.* **17,** 139–154.

Mason, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* **169,** 354–364.

Mason, P. W., Pincus, S., Fournier, M. J., Mason, T. L., Shope, R. E., and Paoletti, E. (1991). Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* **180,** 294–305.

Monath, T. P. (1986). Pathobiology of the flaviviruses. *In* "The Togaviridae and Flaviviridae" (S. Schlesinger and M. J. Schlesinger, Eds.), pp. 375–440. Plenum, New York.

Noonan, C. A., and Butel, J. S. (1978). Transformation by viruses: Simian virus 40 as a model system. *Natl. Cancer Inst. Monogr.* **48,** 227–237.

Oya, A. (1967). The role of mammals as primary and supplementary hosts. *Jpn. J. Med. Sci. Biol. Suppl.* **20,** 26–30.

Phillpotts, R. J., Venugopal, K., and Brooks, T. (1996). Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. *Arch. Virol.* **141,** 743–749.

Pincus, S., Mason, P. W., Konishi, E., Fonseca, B. A. L., Shope, R. E., Rice, C. M., and Paoletti, E. (1992). Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* **187,** 290–297.

Porter, K. R., Kochel, T. J., Wu, S. J., Raviprakash, K., Phillips, I., and Hayes, C. G. (1998). Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. *Arch. Virol.* **143,** 997–1003.

Sasaki, O., Karoji, Y., Kuroda, A., Karaki, T., Takenokuma, K., and Maeda, O. (1982). Protection of pigs against mosquito-borne Japanese encephalitis virus by immunization with a live attenuated vaccine. *Antiviral Res.* **2,** 355–360.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., Silverman, G. J., Lotz, M., Carson, D. A., and Raz, E. (1996). Immuno-

stimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273,** 352–354.

Sato, T., Takamura, C., Yasuda, A., Miyamoto, M., Kamogawa, K., and Yasui, K. (1993). High-level expression of the Japanese encephalitis virus E protein by recombinant vaccinia virus and enhancement of its extracellular release by the NS3 gene product. *Virology* **192,** 483–490.

Schmaljohn, C., Vanderzanden, L., Bray, M., Custer, D., Meyer, B., Li, D., Rossi, C., Fuller, D., Fuller, J., Haynes, J., and Huggins, J. (1997). Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. *J. Virol.* **71,** 9563–9569.

Scherer, W. F., Moyer, J., Izumi, T., Gresser, I., and McCown, J. (1959). Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. *Am. J. Trop. Med. Hyg.* **8,** 698–706.

Shope, R. E. (1980). Medical significance of togaviruses: An overview of diseases caused by togaviruses in man and in domestic and wild vertebrate animals. *In* "The Togaviruses" (R. W. Schlesinger, Ed.), pp. 47–82. Academic Press, New York.

Yamagishi, A. (1989). Japanese encephalitis vaccine. *J. Vet. Med.* **820,** 14–18.

Yamshchikov, V. F., and Compans, R. W. (1993). Regulation of the late events in flavivirus protein processing and maturation. *Virology* **192,** 38–51.

Yoshida, I., Takagi, M., Inokuma, E., Goda, H., Ono, K., Takaku, K., and Oku, J. (1981). Establishment of an attenuated ML-17 strain of Japanese encephalitis virus. *Biken J.* **24,** 47–67.