

# The Anamnestic Neutralizing Antibody Response Is Critical for Protection of Mice from Challenge following Vaccination with a Plasmid Encoding the Japanese Encephalitis Virus Premembrane and Envelope Genes

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**For Japanese encephalitis (JE), we previously reported that recombinant vaccine-induced protection from disease does not prevent challenge virus replication in mice. Moreover, DNA vaccines for JE can provide protection from high challenge doses in the absence of detectable prechallenge neutralizing antibodies. In the present study, we evaluated the role of postchallenge immune responses in determining the outcome of JE virus infection, using mice immunized with a plasmid, pcDNA3JEME, encoding the JE virus premembrane (prM) and envelope (E) coding regions. In the first experiment, 10 mice were vaccinated once (five animals) or twice (remainder) with 100 µg of pcDNA3JEME. All of these mice showed low (6 of 10) or undetectable (4 of 10) levels of neutralizing antibodies. Interestingly, eight of these animals showed a rapid rise in neutralizing antibody following challenge with 10,000 50% lethal doses of JE virus and survived for 21 days, whereas only one of the two remaining animals survived. No unimmunized animals exhibited a rise of neutralizing antibody or survived challenge. Levels of JE virus-specific immunoglobulin M class antibodies were elevated following challenge in half of the unimmunized mice and in the single pcDNA3JEME-immunized mouse that died. In the second experiment, JE virus-specific primary cytotoxic T-lymphocyte (CTL) activity was detected in BALB/c mice immunized once with 100 µg of pcDNA3JEME 4 days after challenge, indicating a strong postchallenge recall of CTLs. In the third experiment, evaluation of induction of CTLs and antibody activity by plasmids containing portions of the prM/E cassette demonstrated that induction of CTL responses alone were not sufficient to prevent death. Finally, we showed that antibody obtained from pcDNA3JEME-immunized mice 4 days following challenge could partially protect recipient mice from lethal challenge. Taken together, these results indicate that neutralizing antibody produced following challenge provides the critical protective component in pcDNA3JEME-vaccinated mice.**

Japanese encephalitis (JE) is a mosquito-borne viral disease causing infection of the central nervous system in humans and equines. It is generally believed that JE virus present in mosquito saliva replicates at or near the bite site and is then transported via the bloodstream into the brain, where it may cause infection and encephalitis. Two major factors have been reported to be important for protection from encephalitis: neutralizing antibody and cytotoxic T lymphocytes (CTLs) specific for JE virus. Passive transfer of monoclonal antibodies to the envelope (E) protein (1, 5, 21), T cells obtained from infected mice (23, 25), and CTLs (26) can protect mice from a lethal challenge. High levels of neutralizing antibody (29) and JE virus-specific T lymphocytes (8) have been detected in JE patients in the convalescent phase.

We have previously studied the immunogenicity of JE gene products in a mouse model using recombinant poxviruses expressing the signal of the premembrane (prM), the prM gene, and the envelope (E) gene. Cells infected with these poxviruses produce subviral extracellular particles (EPs). These subviral particles are similar to the slowly sedimenting hemagglutinin particles produced by cells infected with JE virus, suggesting that the prM, membrane (M), and E proteins in these EPs are comparable to the authentic forms of these proteins (11, 13, 22). Mice immunized with poxvirus-based recombinants encoding the signal-prM-E gene cassette induced high levels of neutralizing antibody and memory CTLs and were protected from lethal challenge (9, 12, 14). However, these mice were not protected from infection by the challenge virus, since high levels of antibody to the nonstructural (NS) proteins were detected in mice surviving challenge (11).

Recently, naked DNA plasmids encoding flavivirus genes have been reported to induce neutralizing antibody and/or protection in mice, using the NS1 gene of JE virus (19) and the prM/E gene of dengue type 2 (6, 30), St. Louis encephalitis (27), and tick-borne encephalitis (31) viruses. We have demonstrated that mice immunized with a plasmid encoding the JE virus signal-prM-E gene cassette (pcDNA3JEME) were also protected from a lethal challenge (15). Interestingly, although

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mice immunized with this DNA produced CTLs that could be detected after *in vitro* stimulation, the levels of neutralizing antibody induced by these DNAs were low or undetectable. Therefore, this system provides a mouse model useful for studying the mechanism of protection against JE. Some other DNA vaccines also have been reported to protect in the absence of neutralizing antibody responses (19, 19a).

In this study, we analyzed the postchallenge immune responses in pcDNA3JEME-immunized mice to elucidate the role of neutralizing antibody and CTLs in protection.

## MATERIALS AND METHODS

**Viruses.** The prototype Nakayama strain of JE virus (20) was used for construction of plasmids, neutralization tests, and spleen cell stimulation, and the virulent Beijing P3 strain (22) was used for mouse challenge studies. Recombinant vaccinia viruses used for infection of target cells in cytotoxicity assays were vP555, encoding the prM, E, and NS1 genes of the Nakayama strain; vP658, encoding E and NS1; vP829, encoding prM and E; and their parent virus, vP410 (11, 22). vP829 and vP410 were also used for preparing antigens in enzyme-linked immunosorbent assay (ELISA). A recombinant vaccinia virus, vP857, encoding the JE virus NS1 and NS2a genes (11) was used for immunochromatological staining assays.

**Plasmids.** The construction of pcDNA3JEME, a pcDNA3-based plasmid encoding the JE virus signal sequence of prM, prM, and E genes, has been described previously (15). In the present study, three new pcDNA3-based plasmids encoding (i) the signal sequence of prM and prM (nucleotides 325 to 882), (ii) the first half of E (nucleotides 883 to 1599), or (iii) the last half of E (nucleotides 1600 to 2382), were constructed essentially by the strategy used for construction of pcDNA3JEME (15). The JE virus cDNA encoding the signal sequence of prM, prM, and E was amplified by PCR with a template plasmid DNA, pARJa (containing Nakayama strain C protein cDNA sequences fused to plasmids PM-7 and PM-6 [24]; GenBank accession no. M73710). The sense primer included an *EcoRI* site, an efficient eukaryotic initiation site (16), and a start codon, followed by the codons encoding (i) Glu-Gly-Ser-Ile-Met-Trp of the prM signal sequence, (ii) the N-terminal six codons of E, or (iii) codons 240 to 245 of E. The antisense primer corresponded to (i) the C-terminal six codons of prM, (ii) codons 234 to 239 of E, or (iii) the C-terminal six codons of E, each of which was adjacent to a termination codon and an *XhoI* site. The amplified cDNA was inserted into the pcDNA3 vector (Invitrogen Corp., San Diego, Calif.) at the *EcoRI/XhoI* site between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from the bovine growth hormone. The constructs encoding prM, the first half of E, and the last half of E were designated pcDNA3JEprM, pcDNA3JEE1/2, and pcDNA3JEE2/2, respectively. The proper insert of the gene cassette in these constructs was confirmed by sequencing using a DNA sequencer (ABI 373A; Applied Biosystems, Chiba, Japan). All plasmid DNAs (pcDNA3, pcDNA3JEME, pcDNA3JEprM, pcDNA3JEE1/2, and pcDNA3JEE2/2) were purified by using a Qiagen plasmid kit (Funakoshi Co. Ltd., Tokyo, Japan) as instructed by the manufacturer and used for immunization of mice.

**Mouse experiments.** Groups of five 4-week-old female ICR mice and three 6-week-old male BALB/c mice were used for evaluating induction of antibody and protective immunity, and groups of two 6-week-old male BALB/c mice were used for evaluating induction of CTLs. Mice were inoculated once or twice at an interval of 2 weeks by intramuscular injections at both thighs with 50  $\mu$ l of immunogens diluted in phosphate-buffered saline (PBS) at each site. Immunogens and doses were pcDNA3JEME at doses of 10 and 100  $\mu$ g and pcDNA3JEprM, pcDNA3JEE1/2, pcDNA3JEE2/2, and the pcDNA3 vector at a dose of 100  $\mu$ g. Two or four weeks after the last immunization, mice were challenged by intraperitoneal (i.p.) injection with 50% lethal doses (10,000 LD<sub>50</sub>) of the Beijing P3 strain of JE virus and observed for 3 weeks. This challenge dose was used throughout the study, including passive transfer experiments. Retro-orbital blood was collected successively from each mouse 2 days before challenge and 2, 4, 6, 8, 11, 14, and 21 days after challenge unless otherwise stated. Individual or pooled serum samples were used for evaluation of antibody. Spleens were collected from immunized BALB/c mice just before challenge and 4, 8, and 14 days after challenge for evaluating induction of primary CTLs, or were collected 2 weeks after immunization for evaluating induction of memory CTLs. Spleen cell suspensions were prepared as previously described (9) and used for cytotoxicity assays (see below).

For passive transfer experiments, female ICR mice were immunized with 100  $\mu$ g of pcDNA3JEME at 4 and 6 weeks of age and challenged with 10,000 LD<sub>50</sub> of the Beijing P3 strain at 8 weeks of age. Sera were collected from these mice just before challenge and 4 and 21 days after challenge. Sera were also collected from unimmunized 8-week-old female ICR mice 4 days after challenge. Immunoglobulin fractions were isolated from pooled sera by precipitation with saturated ammonium sulfate followed by extensive dialysis against PBS. The immunoglobulin fraction corresponding to 0.2 ml of the original serum was transferred by i.p. injection into groups of 10 unimmunized 8-week-old female ICR mice that

had been challenged with 10,000 LD<sub>50</sub> of the Beijing P3 strain 2 days earlier. These mice were observed for 21 days for survival rates.

**Neutralization tests.** Neutralizing antibodies elicited in immunized mice were titrated as previously described (11) except for the inclusion of complement in the virus-antibody mixture. The neutralization titer was expressed as the highest serum dilution yielding a 90% reduction in plaque number.

**ELISA.** Antibodies to JE virus prM/E were measured by ELISA using EPs as antigen as previously described (10). Briefly, EPs were harvested from culture fluid of HeLa cells infected 20 h earlier with vP829, a recombinant vaccinia virus encoding the JE virus (Nakayama strain) prM and E genes, or with vP410, the parent vaccinia virus. After precipitation with 10% polyethylene glycol, EPs were dissociated with 0.1% Triton X-100 and diluted in 0.1 M carbonate buffer (pH 9.6) for sensitization of microplates (Maxisorp; A/S Nunc, Roskilde, Denmark). Sensitized microplates were incubated with mouse serum samples at a 1:100 dilution, with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG; gamma chain specific) or IgM (mu chain specific; EY Laboratories, San Mateo, Calif.) at a dilution of 1:1,000, and then with *p*-nitrophenyl phosphate (1 mg/ml). The difference in absorbance values obtained with the recombinant and control antigens was regarded as a measure of the antibody level specific for prM and E. To minimize interplate variation, all values shown were expressed relative to the reference value obtained with a constant positive control sera, which was included in each assay plate.

**Immunochromatological staining assay.** Antibodies to NS1 were titrated by immunochromatological staining using vP857-infected HeLa cells as antigen. HeLa monolayer cells grown in 96-well microplates were infected with vP857, a recombinant vaccinia virus encoding the JE virus NS1 and NS2a genes, at a multiplicity of infection of 2 PFU/cell. After incubation at 37°C for 6 h, monolayers were rinsed with PBS, fixed with ethanol, dried, and stored at -30°C until use. For testing, monolayers were rehydrated with PBS containing horse serum at 1% and incubated with serial twofold dilutions of test sera. Antigen-antibody reactions were then detected with biotinylated anti-mouse IgG (heavy and light chain specific), the ABC (avidin-biotinylated enzyme complex) reagents, and the VIP substrate (Vector Laboratories, Inc., Burlingame, Calif.). Titers were represented as the maximum dilution that provided stained cells. In a preliminary experiment using sera from unimmunized mice, cells were occasionally stained when incubated with a 1:10 dilution of sera, but no stained cells were observed at a 1:20 dilution. Therefore, we decided a borderline for determination of the antibody to NS1 in mouse sera to be positive at a dilution of 1:20.

**Cytotoxicity assays.** Primary CTLs were assayed without *in vitro* stimulation of spleen cells. Spleen cell suspensions prepared from two BALB/c mice were mixed and washed three times with RPMI 1640 medium containing 10% fetal bovine serum. These cells were distributed in triplicate in 96-well microplates at different cell densities to provide various effector:target (E:T) ratios. The target cells used for these assays were P815 mastocytoma cells infected with vP829 or vP410 at a multiplicity of infection of 10 PFU/cell 15 to 20 h before the assay. All target cells were labeled with Na<sup>51</sup>CrO<sub>4</sub>, washed, and distributed evenly at 1 × 10<sup>5</sup> or 2 × 10<sup>5</sup> viable cells per well into microplates containing effector cells. The plates were incubated for 5 to 6 h at 37°C, and <sup>51</sup>Cr release into the supernatant was measured in a gamma counter. Percent specific lysis was calculated by the following formula: 100 × (experimental release - minimum release)/(maximum release - minimum release), where the maximum release was obtained by lysing all the target cells with polyoxyethylene(12)tridecyl ether (Renex; Ruger Chemical Co., Irvington, N.J.) and the minimum release was obtained with target cells incubated alone in RPMI 1640-10% fetal bovine serum.

Assays for secondary CTLs and the stimulation of spleen cells with the Nakayama strain of JE virus *in vitro* were performed as previously described (15). Briefly, spleen cells were stimulated by incubation with the live JE virus antigen at 37°C for 6 days. Stimulated cells were incubated with <sup>51</sup>Cr-labeled target cells for 5 to 6 h at 37°C. The target cells used for these assays were P815 mastocytoma cells infected with vP555, vP658, vP829, or vP410.

## RESULTS

**Postchallenge antibody responses in mice immunized twice with pcDNA3JEME.** Two groups of five ICR mice were immunized with 100  $\mu$ g of pcDNA3JEME or 100  $\mu$ g of the pcDNA3 vector twice at 4 and 6 weeks of age and then challenged with 10,000 LD<sub>50</sub> of the Beijing P3 strain of JE virus at 8 weeks of age. Prechallenge sera were collected 2 days before challenge (defined as day -2) and examined for neutralizing antibody. Following challenge, mice were observed for 3 weeks, and postchallenge sera were collected on days 4, 8, and 21 from all the surviving mice. The results in Table 1 show that mice immunized with pcDNA3JEME had individual antibody titers at 1:10 to 1:20 on day -2. After challenge, neutralization titers were elevated to 1:160 to 1:320 on day 4 and 1:320 to 1:1280 on day 8, and all mice survived for 21 days. Neutralization titers on day 21 were similar to those on day 8. Although a small number

TABLE 1. Postchallenge immune responses in individual ICR mice immunized twice with pcDNA3JEME

Immunogen <sup>b</sup>	Mouse no.	Neutralization titer <sup>a</sup>				Anti-NS1 antibody <sup>d</sup> titer	Outcome on day 21 (day of death)
		Day -2 <sup>c</sup>	Day 4	Day 8	Day 21		
pcDNA3JEME	1	1:10	1:320	1:640	1:640	1:640	Alive
	2	1:10	1:160	1:640	1:1,280	1:640	Alive
	3	1:10	1:160	1:320	1:640	1:320	Alive
	4	1:20	1:160	1:640	1:640	1:320	Alive
	5	1:10	1:160	1:1,280	1:640	1:640	Alive
pcDNA3	6	<1:10	1:10	NA <sup>e</sup>	NA	NA	Dead (7)
	7	<1:10	<1:10	1:10	NA	NA	Dead (10)
	8	<1:10	1:10	1:20	NA	NA	Dead (9)
	9	<1:10	1:10	NA	NA	NA	Dead (7)
	10	<1:10	<1:10	NA	NA	NA	Dead (6)

<sup>a</sup> Highest serum dilution yielding a 90% reduction in plaque number.

<sup>b</sup> Groups of five female ICR mice were inoculated with 100 µg of pcDNA3JEME or pcDNA3 at 4 and 6 weeks of age and then challenged at 8 weeks of age.

<sup>c</sup> Days relative to challenge. Day -2 denotes 2 days before challenge.

<sup>d</sup> Titrated by immunochemical staining assay using vP857-infected HeLa cells as antigen.

<sup>e</sup> NA, serum was not available.

of mice were used in each experiment, the postchallenge neutralizing antibody responses observed in the present study were consistent with those obtained in pilot experiments (data not shown) and in our previous study on duration of protective immunity induced by pcDNA3JEME (15). Mice inoculated with the pcDNA3 vector did not have neutralizing antibody before challenge. Neutralizing antibody titers did not exceed 1:10 on day 4 and 1:20 on day 8, and all of these animals died within 10 days of challenge.

All five pcDNA3JEME-immunized mice had anti-NS1 antibody on day 21 (Table 1). Since the immunogen, pcDNA3JEME, does not contain the NS1 gene, the presence of antibody to NS1 indicates replication of challenge virus in these mice.

**Postchallenge antibody responses in mice immunized once with pcDNA3JEME.** We previously reported lower protective indices with a single immunization with 10 or 100 µg of pcDNA3JEME than with two immunizations (15). To analyze postchallenge antibody responses at subprotective doses, three groups of five ICR mice immunized once with 100 or 10 µg of pcDNA3JEME or PBS at 4 weeks of age were challenged at 8 weeks of age. Sera were collected 2 days before challenge and 2, 4, 6, 8, 11, 14, and 21 days after challenge and were examined individually for neutralizing antibody (Fig. 2), IgG (Fig. 3), and IgM (Fig. 4) antibodies to prM/E and for antibodies to NS1 (Fig. 5).

Figure 1 shows the survival data for these mice. Four of five mice immunized with 100 µg of pcDNA3JEME survived, but all mice immunized with 10 µg of pcDNA3JEME or PBS died during the 21-day observation period. All mice that survived for 21 days maintained a healthy appearance throughout the observation period.

Differences in the kinetics of the appearance of neutralizing antibody among these groups (Fig. 2) were predictive of survival (Fig. 1). Neutralization titers before challenge were undetectable (<1:10) in all mice but one in the pcDNA3JEME 100-µg group, which showed a titer of 1:10 (Fig. 2). Four days after challenge, three of the five mice immunized with 10 µg showed neutralization titers of 1:80 or greater, and all three of these mice survived for 21 days. The remaining two mice showed a neutralization titer of 1:20 on day 4, and one of these mice died on day 16. Levels of neutralizing antibody in the mouse that died of challenge (Fig. 2) were 1:20 or less within the survival period, whereas those in the mouse that survived

for 21 days were 1:40 or greater on day 8 and thereafter. In other groups (10 µg of pcDNA3JEME and PBS), the neutralization titers did not exceed 1:20 throughout the survival period and all the mice died.

Figures 3 and 4 show the appearance of IgG and IgM antibodies to prM/E in these mice. Overall, the appearance of IgG antibody (Fig. 3) followed the kinetics observed for neutralizing antibody (Fig. 2). Two mice immunized with 100 µg of pcDNA3JEME showed IgG antibody levels of over 1.2 on day 4 and maintained these levels until day 21 (Fig. 3). However, three other mice in the 100-µg group showed low levels of IgG antibodies to prM/E (less than 0.5) on day 4 or later. Two of these three mice survived challenge, whereas one died on day 16. All the mice immunized with 10 µg of pcDNA3JEME or PBS alone showed ELISA levels of less than 0.4 throughout the survival period.

Time course of the appearance of IgM antibody to prM/E showed a maximum level on day 4 in most of the mice that survived more than 8 days after challenge (Fig. 4). Among the 10 mice inoculated with 10 µg of pcDNA3JEME or PBS, which all died following challenge, 5 showed levels of more than 0.2 on day 4, whereas all the 4 animals which were immunized with 100 µg of pcDNA3JEME and survived challenge showed levels

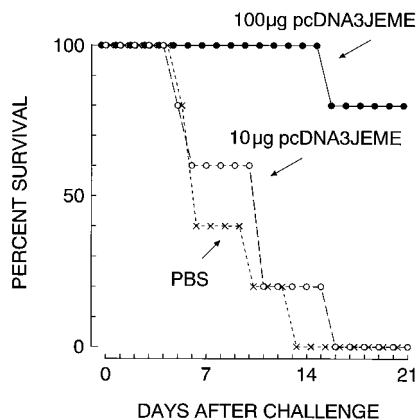


FIG. 1. Survival of ICR mice after challenge in the one-immunization protocol. Three groups of five mice were inoculated with 100 or 10 µg of pcDNA3JEME or PBS at 4 weeks of age and challenged at 8 weeks of age.

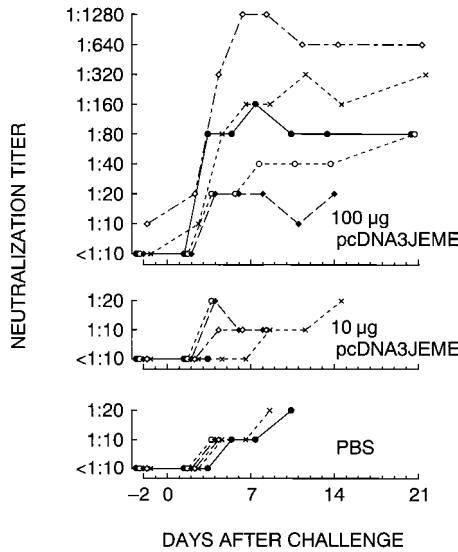


FIG. 2. Time course of neutralizing antibody titers in individual mice after challenge in the one-immunization protocol. Three groups of five ICR mice were inoculated with 100 or 10 µg of pcDNA3JEME or PBS at 4 weeks of age and challenged at 8 weeks of age. Sera collected 2 days before challenge and 2, 4, 6, 8, 11, 14, and 21 days after challenge were used for the test. Neutralization titers were represented as the highest serum dilution yielding a 90% reduction in plaque number. The symbols used in this figure are likewise used in Fig. 3 to 5 for representing data obtained from the same mice in each group.

of less than 0.2. The one mouse that was immunized with 100 µg of pcDNA3JEME and died showed an IgM antibody level of more than 0.3 on day 4, suggesting that this mouse was not effectively primed by one inoculation with 100 µg of pcDNA3JEME.

Figure 5 shows the appearance of antibodies to NS1 in the three groups of mice. The data represent the dilution of sera that reacted with NS1 in an immunochemical staining assay.

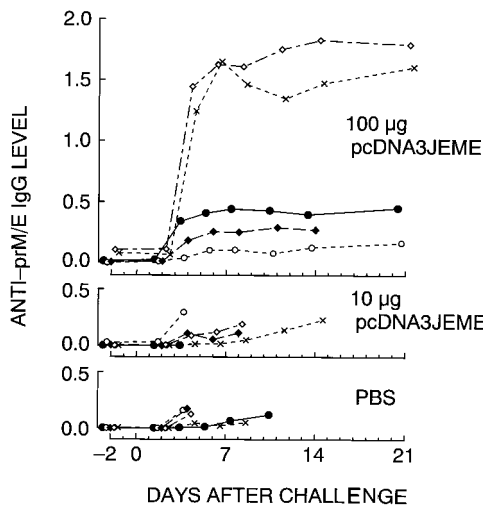


FIG. 3. Time course of IgG antibody to prM/E in individual mice after challenge in the one-immunization protocol. Three groups of five ICR mice were inoculated with 100 or 10 µg of pcDNA3JEME or PBS at 4 weeks of age and challenged at 8 weeks of age. Sera collected 2 days before challenge and 2, 4, 6, 8, 11, 14, and 21 days after challenge were examined. Antibody levels were measured by ELISA and presented as relative values (see Materials and Methods for details). The symbols used in this figure are likewise used in Fig. 2, 4, and 5 for representing data obtained from the same mice in each group.

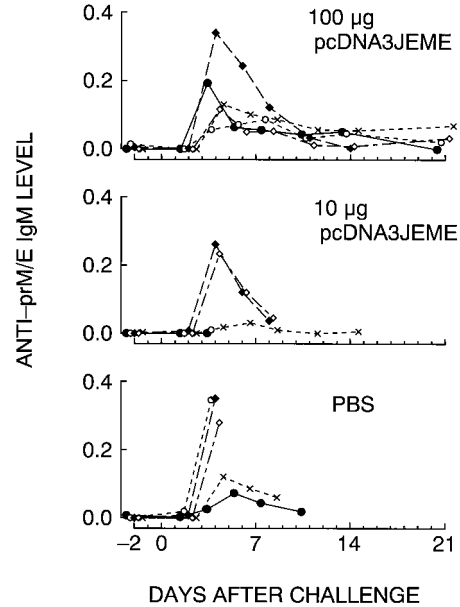


FIG. 4. Time course of IgM antibody to prM/E in individual mice after challenge in the one-immunization protocol (see the legend to Fig. 3 for details).

This assay was based on an indirect staining protocol that should detect both IgG and IgM class antibodies. Since NS1 is not included in the vaccine DNA, the magnitude of response to NS1 can be used as an indicator of challenge virus replication (11). The antibody to NS1 appeared on day 2 after challenge in two of the five mice inoculated with PBS, whereas the antibody to NS1 was first detected on day 4 in mice immunized with 100 or 10 µg of pcDNA3JEME. All mice, except one immunized with 10 µg of pcDNA3JEME that died before antibody to NS1 was detected, showed increasing levels of antibody to NS1 throughout the observation period. In some of the animals that

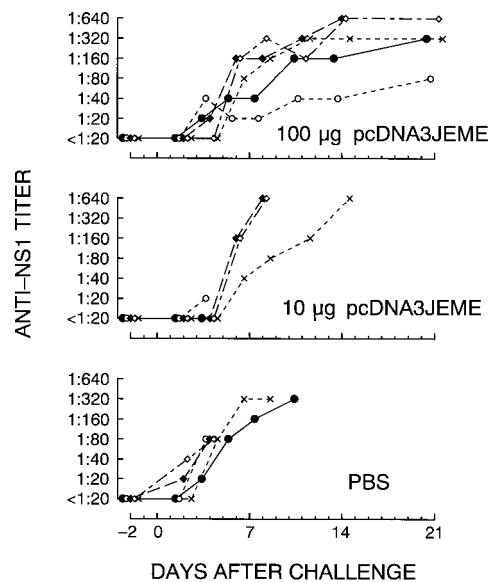


FIG. 5. Time course of antibody to NS1 in individual mice after challenge in the one-immunization protocol (see the legend to Fig. 3 for details). Antibody titers were measured by an immunochemical staining assay (see Materials and Methods for details).



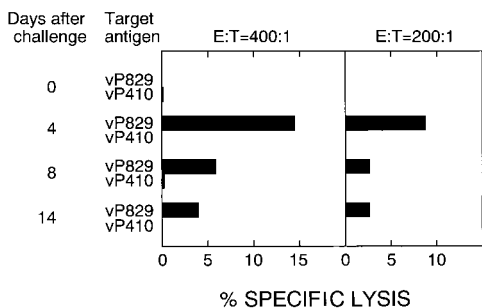


FIG. 6. Primary CTL activity in pcDNA3JEME-immunized BALB/c mice after challenge. Four groups of two mice were immunized with 100 µg of pcDNA3JEME at 6 weeks of age and challenged at 9 weeks of age. Spleen cells were collected just before challenge (day 0) and 4, 8, and 14 days after challenge. Cytotoxic activities against P815 cells infected with vP829 or vP410 were measured at E:T ratios of 400:1 and 200:1 by the standard chromium release method (see Materials and Methods for details). vP829 is a recombinant vaccinia virus encoding prM and E; vP410 is a parent vaccinia virus with no JE virus genes.

died within 10 days of challenge, there was a rapid rise in antibody to NS1 immediately preceding death (Fig. 5). The one mouse immunized with 100 µg of pcDNA3JEME that had a low level of neutralizing antibody but survived showed low levels of anti-NS1 antibody throughout the observation period, suggesting very limited replication of the challenge virus in this animal.

**Postchallenge CTL responses.** The primary CTL responses induced by challenge were examined in spleen cells of immunized mice. Groups of two male BALB/c mice were immunized once with 100 µg of pcDNA3JEME at 6 weeks of age and challenged at 9 weeks of age. Spleen cells were collected in different postchallenge days and examined for CTL activity without *in vitro* stimulation. In the first experiment using spleen cells obtained before challenge and on days 1, 2, and 3 after challenge, no CTL activity was detected (data not shown). In the second experiment using spleen cells obtained before challenge and on days 4, 8, and 14 after challenge (Fig. 6), approximately 10 to 15% of specific lysis was observed against target cells infected with vP829 in samples obtained on day 4. Spleen cells did not lyse target cells infected with vP410. The cytotoxicity levels obtained in mice on days 8 and 14 were lower than those obtained on day 4. In contrast, cells collected on day 0 (just before challenge) did not lyse targets infected with vP829. These results indicate that active CTLs were present in the immunized mice 4 days after challenge.

**Postchallenge immune responses in mice immunized with plasmids encoding a subset of the prM/E cassette.** To further elucidate the role of antibody and CTLs in protection, we constructed three additional pcDNA3-based plasmids: pcDNA3JEprM, encoding the signal sequence of prM and prM; pcDNA3JEE1/2, encoding the first half of E; and pcDNA3JEE2/2, encoding the last half of E. Five groups of five BALB/c mice were immunized with 100 µg of each of these plasmids, pcDNA3JEME, or pcDNA3 at 6 and 8 weeks of age. Two of these five mice were used to examine memory CTLs present at 10 weeks of age, and the remaining three mice were challenged at 12 weeks of age. Figure 7 shows the cytotoxic activities of spleen cells isolated from these groups of mice prior to challenge. Mice immunized with pcDNA3JEE1/2 showed approximately 10 to 30% of specific lysis against target cells expressing E (vP555, vP658, and vP829) at E:T ratios of both 400:1 and 200:1, but no cytotoxic activities were observed against control target cells (vP410). In mice immunized with pcDNA3JEprM, cytotoxic levels observed against target cells

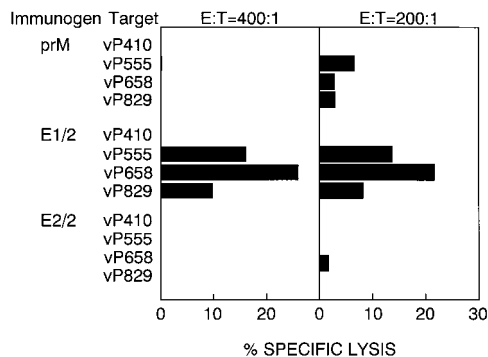


FIG. 7. Memory CTLs induced in BALB/c mice immunized with plasmids encoding the JE virus prM (prM), the first half of E (E1/2), or the second half of E (E2/2). Mice were immunized with 100 µg of each plasmid at 6 and 8 weeks of age. Spleen cells were collected at 10 weeks of age and stimulated by incubation with live JE virus for 6 days at 37°C. Cytotoxic activities were measured at E:T ratios of 400:1 and 200:1 by the standard chromium release method (see Materials and Methods for details). The target cells were P815 cells infected with a recombinant vaccinia virus (vP555 encoding prM, E, and NS1; vP658 encoding E and NS1; or vP829 encoding prM and E) or the parent vaccinia virus, vP410.

expressing prM (vP555 and vP829) were quite low, and the value obtained with the vP829 target cells was identical to the value obtained with vP658 target cells, which do not express prM. These data show that memory CTLs could be detected in mice immunized with the first half of E but not in mice immunized with prM or the second half of E, indicating that the CTL epitope(s) is located only in the first half of the E region.

Figure 8 shows the survival curve and the course of antibody levels. Sera pooled from three mice were examined for neutralizing antibody and IgG and IgM antibodies to prM/E. Consistent with the previous results, pcDNA3JEME induced protection and high levels of postchallenge antibody responses, but pcDNA3 did not. All mice immunized with pcDNA3JEE1/2, which induced CTLs, died of challenge. Only low levels of neutralizing antibody (1:20 on day 4) and the IgG

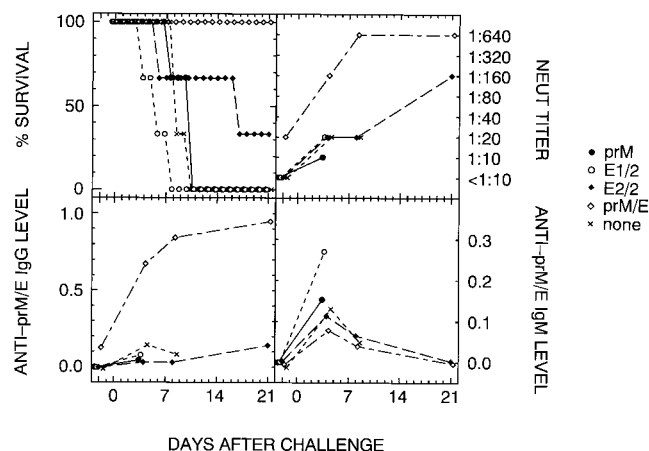


FIG. 8. Survival curves and time courses of postchallenge immune responses in BALB/c mice immunized with plasmids encoding a subset of the JE virus prM/E cassette. Mice were immunized with 100 µg of pcDNA3JEprM (prM), pcDNA3JEE1/2 (E1/2), pcDNA3JEE2/2 (E2/2), pcDNA3JEME (prM/E), or pcDNA3 (none) at 6 and 8 weeks of age and were challenged at 12 weeks of age. Sera collected 2 days before challenge and 4, 8, and 21 days after challenge were pooled and measured for neutralizing (NEUT) antibody and IgG and IgM antibodies to prM/E by ELISA (see Materials and Methods for details). In a group of mice immunized with pcDNA3JEME, one mouse died accidentally upon being given anesthesia, and all evaluations were done with two mice.

TABLE 2. Partial protection of mice passively transferred with antibody produced in pcDNA3JEME-immunized mice after challenge

Donor mice		Neutralization titer of pooled sera used for transfer	Protection (no. of surviving mice/total no. of mice)
Immune status <sup>a</sup>	Time (day postchallenge) of serum collection <sup>b</sup>		
Immunized	0	1:10	0/10
	4	1:320	3/10
	21	1:1,280	4/10
Unimmunized	4	1:10	0/10

<sup>a</sup> Four-week-old female ICR mice were immunized twice with 100  $\mu$ g of pcDNA3JEME at an interval of 2 weeks or were unimmunized.

<sup>b</sup> At 8 weeks of age, mice were challenged with 10,000 LD<sub>50</sub> of the P3 strain. Day 0 denotes just before challenge.

antibody to prM/E, as well as a relatively high level of IgM antibody, were induced in these mice. pcDNA3JEprM also induced no protection and low levels of IgG antibody response to prM/E. Only one of three mice immunized with pcDNA3JEE2/2 survived, and this animal had a neutralization titer of 1:160 on day 21. These results suggest that the induction of memory CTLs to the first half of the E region was not sufficient to protect mice from lethal challenge.

**Passive protection by transfer of postchallenge serum.** To clarify the role of neutralizing antibody produced following challenge in protection, immunoglobulin fractions of the sera collected from mice immunized by various procedures were transferred to ICR mice 2 days following inoculation with a lethal dose of JE virus. The tested sera included the sera pooled from pcDNA3JEME-immunized mice, sera pooled from the same mice at 4 or 21 days postchallenge, and sera pooled from unimmunized mice 4 days after challenge. The levels of neutralizing antibody present in these preparations and the survival data are shown in Table 2. Three and four of ten mice which were given antibody obtained from immunized mice 4 and 21 days after challenge, respectively, survived for 21 days, whereas no protection was observed by transfer of antibody obtained from prechallenge sera of immunized mice or postchallenge sera of unimmunized mice. Although the levels of protection were low, we concluded that the antibody induced a partial protection, since the Beijing P3 strain is uniformly lethal in mice at the dose used in this study (10,000 LD<sub>50</sub>).

## DISCUSSION

This study demonstrates that neutralizing antibody produced in response to challenge provides the critical protective component in pcDNA3JEME-immunized mice. A remarkable difference in postchallenge neutralization titer was observed between mice that survived and mice that died of lethal challenge. Although some of the surviving mice had detectable levels of neutralizing antibody before challenge, these animals were not protected from infection by the challenge virus, since antibodies to the viral NS1 protein (not encoded by the DNA vaccine) were detected after challenge. Primary CTLs could also be detected in pcDNA3JEME-vaccinated mice. However, these CTLs do not appear to be the critical protective component, since mice which were immunized with a shorter plasmid and developed CTLs but not neutralizing antibody were not protected from challenge. On the other hand, partial protection was observed when antibody collected from pcDNA

3JEME-immunized mice 4 days after challenge was administered to naive mice 2 days after JE challenge. Thus, pcDNA3JEME protects mice from disease but not infection.

It has been reported that mice were partially protected from challenge by passive transfer of hyperimmune or monoclonal anti-JE virus antibodies, either before or after lethal challenge (5, 28, 32). We also observed protection in our passive transfer experiments and assume that the protection was incomplete, since insufficient levels of antibody were present or maintained in the recipient mice. Specifically, the transferred antibody that conferred partial protection had a titer of 1:320 to 1:1,280 at the time of injection; thus, the final neutralization titer in the recipient was expected to be in the range of only 1:32 to 1:128, assuming a serum volume of 2 ml in mice of this age. Moreover, passively transferred antibodies may not have a long circulating time in mice (7), so our partial protection data may be consistent with the circulating levels of antibody at the time of virus multiplication.

In our mouse model, the challenge virus was injected by an i.p. route, simulating the primary (peripheral site) and secondary (central nervous system) growth of virus, which results in encephalitis following arthropod-mediated transmission. Thus, in both natural infection and experimental challenge, virus replication at peripheral sites provides progeny virus capable of stimulating the immune response, as well as blood-borne virus that can induce the signs and symptoms of the disease. Based on our estimates of the levels of neutralizing antibody circulating in mice following challenge (see above), the titers of antibody present in the sera of pcDNA3JEME-vaccinated mice following challenge should have been sufficient to provide protection from encephalitis. On the other hand, unimmunized mice (or control plasmid-immunized mice) did not produce sufficient neutralization titers in the face of challenge and were not protected. Thus, a rapid postchallenge elevation of neutralizing antibody in response to virus replication, due to the presence of memory B and helper T cells in mice inoculated with pcDNA3JEME, appears to be the main mechanism of protection by pcDNA3JEME.

Although two immunizations with 100  $\mu$ g of pcDNA3JEME provided mice with postchallenge neutralization titers sufficient for protection, single immunizations with 100 or 10  $\mu$ g of pcDNA3JEME elicited variable outcomes. These data support the importance of a minimum level of preexisting B cells capable of producing protective levels of neutralizing antibodies following challenge. Thus, in the once-immunized mice with insufficient B-cell priming, the rate of virus replication and spread was able to overwhelm the rising levels of neutralizing antibodies.

The CTL activity induced by immunization twice with 100  $\mu$ g of pcDNA3JEE1/2 did not correlate to the protection under the conditions used in our experiments. As stated above, this result suggests that postchallenge antibody levels are the primary mediators of protection. However, passive transfer with virus-specific CTLs obtained from JE virus-infected mice can protect mice from encephalitis under some conditions (26), suggesting that CTLs specific for other epitopes, or larger numbers of CTLs, could be important for protection from JE. For the dengue viruses, a relatively large number of CTL epitopes have been identified in NS proteins (2, 18), although their ability to protect animals from disease has not yet been determined. Immunization with plasmid DNA encoding portions of the JE virus polyprotein may prove a particularly effective way to determine a protective role of individual CTL epitopes encoded by other regions of the genome.

Passive protection against flavivirus infection in mice has been reported with IgM class antibodies raised in response to

viral infection (23) and with monoclonal antibodies to NS1 (3). In the present study, since all postchallenge samples collected from protected mice contained both specific IgM and IgG antibodies, we cannot state with certainty which isotypes of antibody were critical for protection. However, sera that had a maximum level of IgM antibody on day 4 did not show high neutralizing activity (<1:20), and mice with this serological profile did not survive challenge. A role for antibody to NS1 in protection cannot be ruled out in our studies; however, antibodies to NS1 rose similarly in all groups, in contrast to the enhanced secondary responses to prM/E in the DNA-vaccinated animals that were protected.

In conclusion, the critical components for protection of mice from JE appear to be preexisting memory B and T cells which produce sufficient levels of neutralizing antibodies following virus encounter. The presence of detectable prechallenge neutralizing antibody was not always required for protection from death. These conclusions may be applicable to humans, swine, and horses as well. In the case of human infection, the existing JE vaccine, which is considered to provide protection from challenge, does not stimulate 100% seroconversion in vaccinated populations (4). Moreover, JE is considered to be endemic in Japan, where most of the population receives this vaccine in childhood. Although most healthy adults below the age of 60 do not receive booster immunizations, disease is very rare. One explanation for this finding is that these individuals are naturally boosted by periodic bites from JE virus-infected mosquitoes (28). In a very small study, we found evidence of this type of boosting, due to the presence of antibodies to NS1 in two of three Japanese who had been vaccinated and lived in Japan (8). Thus, it seems highly probable that secondary neutralizing antibody responses can protect humans from the disease following the bite by infected mosquitoes. In general, it is considered that the inactivated vaccine induces neutralizing antibody that protects humans from infection. The protective immunity against infection might be provided when high antibody levels are maintained; however, it is much more likely that this vaccine induces memory cells that produce a secondary immune response following infection and that the produced antibodies limit infection and stop disease. In this sense, the current inactivated JE vaccines protect by the same mechanism as tick-borne encephalitis vaccines, in that they do not provide protection from infection (17). The continued elucidation of mechanisms for protection against flavivirus disease will be important for development of new types of vaccines.

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