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Induction of Protective Immunity against Japanese Encephalitis in Mice by Immunization with a Plasmid Encoding Japanese Encephalitis Virus Premembrane and Envelope Genes

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A DNA vaccine plasmid containing the Japanese encephalitis (JE) virus premembrane (prM) and envelope (E) genes (designated pcDNA3JEME) was evaluated for immunogenicity and protective efficacy in mice. Two immunizations of 4-week-old female ICR mice with pcDNA3JEME by intramuscular or intradermal injections at a dose of 10 or 100 μg per mouse elicited neutralizing (NEUT) antibodies at titers of 1:10 to 1:20 (90% plaque reduction), and all immunized mice survived a challenge with 10,000 50% lethal doses of the P3 strain of JE virus. A single immunization with 100 μg of pcDNA3JEME did not elicit detectable NEUT antibodies but induced protective immunity. Spleen cells obtained from BALB/c mice immunized once with 10 or 100 μg of pcDNA3JEME contained JE virus-specific memory cytotoxic T lymphocytes (CTLs). BALB/c mice maintained detectable levels of memory B cells and CTLs for at least 6 months after one immunization with pcDNA3JEME at a dose of 100 μg. The CTLs induced in BALB/c mice immunized twice with 100 μg of pcDNA3JEME were CD8 positive and recognized mainly the envelope protein. These results indicate that pcDNA3JEME has the ability to induce a protective immune response which includes JE virus-specific antibodies and CTLs.

One of the recent promising strategies in protection from viral diseases is the induction of protective immunity by the expression of subsets of viral genes in the vaccinated host. This strategy can eliminate immune responses to unneeded or adventitious antigens present in inactivated virus vaccine preparations and may provide improved safety relative to live attenuated virus vaccines. The introduction of subsets of viral genes into a vaccinee can be accomplished with a recombinant virus (32) or with naked DNA molecules designed to express the genes in the cells of the host (22).

We have studied Japanese encephalitis (JE) as a model for understanding the immunogenicity and protective efficacy conferred on murine, porcine, and human hosts by different flavivirus gene products. In these studies, we showed that recombinant poxviruses carrying the signal sequence for the premembrane (prM), the prM gene, and the envelope (E) gene express proper forms of the prM and E proteins in infected cells and that infected cells release these viral proteins in a particulate form (15, 25). These extracellular particles are morphologically and biochemically similar to the authentic subviral particles, so-called slowly sedimenting hemagglutinin, released from JE virus-infected cells (17). The similarity of these genetically engineered products to natural virus particles is consistent with our early work showing the excellent performance of vaccinia virus-based vaccines specific for these particles in mice (15, 25). Furthermore, a recombinant poxvirus carrying the same signal sequence-prM-E cassette but based on a highly attenuated vaccinia virus strain (NYVAC) induced high levels of neutralizing (NEUT) antibodies (16) and specific cytotoxic T lymphocytes (CTLs) in mice (13) and protected mice from lethal challenge and swine from viremia (16). However, this NYVAC-based recombinant poxvirus did not induce NEUT antibodies to JE virus in vaccinia virus-preimmune vaccinees in a clinical phase I trial, although it did elicit anti-JE virus antibodies in vaccinia virus-naive vaccinees (14).

The adverse effect of antivector immunity to the immunogenicity of the products specified by the vector has been pointed out with several systems (2, 8, 33) and may cause significant problems for the viral vector-based strategy, especially in long-lived species, such as humans. Naked DNA vaccines, which do not suffer from the problem of antivector immunity, recently have been developed and tested for a variety of viral pathogens (3, 31, 34–36). Recently, naked DNA vaccine candidates have been reported for two flavivirus diseases. Work with St. Louis encephalitis showed that a plasmid carrying the prM and E genes could induce partial protection in mice, but induction of NEUT antibodies and CTLs was not demonstrated (28). Another plasmid containing the prM gene and part of the E gene of dengue type 2 virus induced NEUT antibodies, but protection was not demonstrated (12). In this report, we studied the immunogenicity and protective efficacy of plasmid DNA containing the signal sequence-prM-E cassette of JE virus genes that we had identified to be the most effective immunogen in poxvirus-based recombinant viral vaccines for JE.

MATERIALS AND METHODS

Construction of plasmids. The JE virus cDNA containing the prM signal sequence, the prM gene, and the E gene was amplified by PCR with DNA template plasmid pARJa (containing Nakayama strain C protein cDNA sequences fused to plasmids PM-7 and PM-6 [26]; GenBank accession no. M73710). The sense

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primer included an *Eco*RI site, an efficient eukaryotic initiation site (19), and a start codon, followed by the codons encoding Glu-Gly-Ser of the prM signal sequence. The antisense primer corresponded to the C-terminal six codons of the E gene, a termination codon, and an *Xho*I site. To facilitate "error-free" amplification, the selected JE virus coding region was amplified in two portions, which were combined by use of an artificial *Eco*RV site that was added within the coding region (codons 67 and 68 of the E protein) without changing the encoded amino acid sequence. The amplified cDNA was inserted into the pcDNA3 vector (Invitrogen Corp., San Diego, Calif.) at the *Eco*RI/*Xho*I site between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from the bovine growth hormone. The construct was designated pcDNA3JEME. Proper insertion of the gene cassette in pcDNA3JEME was confirmed by sequencing with a DNA sequencer (ABI 373A; Applied Biosystems, Chiba, Japan). pcDNA3JEME DNA was purified with a Qiagen Plasmid Kit (Funakoshi Co. Ltd., Tokyo, Japan) following the manufacturer's instructions and was used for immunization of mice.

Viruses. The prototype Nakayama strain of JE virus (23) was used in all in vitro studies, including NEUT tests, spleen cell stimulation, and cytotoxicity assays. The Nakayama strain, which exhibits low lethality in many strains of mice, was also used to "vaccinate" mice. The Beijing P3 strain of JE virus, which is reproducibly virulent in mice over 6 weeks of age, was used for mouse challenge studies (25). Recombinant vaccinia viruses used for infection of target cells in cytotoxicity assays were vP555, carrying the prM, E, and NS1 genes of the Nakayama strain of JE virus; vP658, carrying the E and NS1 genes; vP829, carrying the prM and E genes; and their parent virus, vP410 (15).

Mouse experiments. Groups of five 4-week-old female ICR mice were used for evaluating the induction of NEUT antibodies and protective immunity, and groups of two 6-week-old male BALB/c mice were used mainly for evaluating the induction of CTLs. Mice were immunized with pcDNA3JEME at doses of 0.1 to 100 μg, pcDNA3 at a dose of 100 μg, or phosphate-buffered saline (PBS) once or twice at an interval of 2 weeks. The injection route was intramuscular (i.m.) at both thighs or intradermal (i.d.) at the base of the tail. At 2 or 3 weeks after immunization, the ICR mice were bled retro-orbitally, and serum samples were isolated from blood, pooled, and used for evaluation of antibody. The ICR mice were also challenged by intraperitoneal (i.p.) injection with 10,000 50% lethal doses (LD_{50}) of the P3 strain of JE virus and observed for 3 weeks. Postchallenge blood was collected from mice that survived the challenge. Spleen cell suspensions were prepared from BALB/c mice as previously described (13) and stimulated with JE virus for cytotoxicity assays (see below). For examination of the duration of NEUT antibodies and memory B cells and CTLs, BALB/c mice that had received one inoculation with pcDNA3JEME at a dose of 100 μg were kept for 1 to 6 months before sample collection.

NEUT tests. Specific antibodies elicited in immunized mice were evaluated by NEUT tests as previously described (15). The NEUT titer was expressed as the serum dilution yielding a 90% reduction in plaque number.

Cytotoxicity assays. Stimulation of spleen cells with JE virus in vitro and cytotoxicity assays were performed as previously described (13) with some modifications. Spleen cells (4×10^6) were stimulated by incubation with live JE virus antigen at a final dilution of 1:8 in 2 ml of RPMI 1640 medium containing 10% fetal bovine serum (RPMI–10% FBS) per well of 24-well microplates at 37°C for 6 days. The live virus antigen used was clarified culture fluid harvested from infected C6/36 cell cultures and contained a titer of approximately 2×10^8 PFU/ml in the undiluted stock, as titrated on Vero cell cultures. The control antigen used was culture fluid from mock-infected C6/36 cell cultures. Both live virus and control antigens were used at 1:8 dilutions. Following the 6-day stimulation step, the cells were washed three times with RPMI–10% FBS and 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 primary mouse kidney (PMK) cells prepared from kidneys of BALB/c mice or P815 mastocytoma cells. PMK cells were infected with JE virus at a high multiplicity of infection (approximately 100 to 200 PFU/cell) or mock infected 17 to 18 h before the assay, and P815 cells were infected with vP829 or vP410 at a multiplicity of infection of 10 PFU/ml or mock infected 15 to 20 h before the assay. For target protein analysis, P815 cells infected with vP555 and vP658 were also used. All target cells were labeled with $Na^{51}CrO_4$, washed, and distributed evenly at 1×10^3 or 2×10^3 viable cells per well into microplates containing effector cells. The plates were incubated for 5 to 6 h at 37°C, and ^{51}Cr release into the supernatant was measured in a gamma counter. Percent specific lysis was calculated with the following formula: $100 \times [(\text{experimental release} - \text{minimum release})/(\text{maximum release} - \text{minimum release})]$; the maximum release was obtained by lysing all the target cells with Renex, and the minimum release was obtained with target cells incubated alone in RPMI–10% FBS.

Cell depletion assays. Cell depletion tests were performed as previously described (13). Briefly, cells stimulated with JE virus were incubated with antibodies to CD3, CD4, and CD8 at dilutions of 1:5 to 1:100 at 4°C for 30 min. These cells were then treated with rabbit complement at a 1:10 dilution at 37°C for 1 h and used in cytotoxicity assays. Cytotoxicity was compared with that obtained with JE virus-stimulated cells treated only with complement and with JE virus-stimulated and mock-stimulated cells without treatment.

TABLE 1. Immunogenicity of pcDNA3JEME in ICR mice with two immunizations

Immunogen[a]	Route	Dose at wk[b]		NEUT titer[c] at wk[b]			Survival[d]
		4	6	6	8	11	
pcDNA3JEME	i.m.	100 μg	100 μg	<1:10	1:20	1:640	5/5
pcDNA3JEME	i.m.	10 μg	10 μg	<1:10	1:10	1:320	5/5
pcDNA3JEME	i.d.	100 μg	100 μg	<1:10	1:10	1:160	5/5
pcDNA3JEME	i.d.	10 μg	10 μg	<1:10	1:20	1:640	5/5
PBS	i.m.			<1:10	<1:10	NA[e]	0/5
JE virus[f]	i.p.	5×10^6 PFU	None	1:80	1:160	≥1:1,280	2/2

[a] Each immunogen was given to five female ICR mice at the ages indicated, and the mice were challenged at week 8.

[b] Ages of the mice at the time of inoculations or serum collections; 11 weeks indicates 3 weeks postchallenge.

[c] Represented as the serum dilution yielding a 90% reduction in plaque number.

[d] Number of surviving mice/total number of mice tested 3 weeks after challenge with 10,000 LD_{50} of the P3 strain of JE virus.

[e] NA, serum was not available.

[f] The immunogen given at 4 weeks consisted of the live Nakayama strain of JE virus at a dose that killed three of five mice. Therefore, all serological and challenge data were derived from the remaining two mice.

RESULTS

Proper expression of pcDNA3JEME. COS7 and Vero cells were transfected with pcDNA3JEME by use of liposomes (Lipofectin; Life Technologies Inc., Gaithersburg, Md.) or lipopolyamine (Transfectam; Biosepra, Villeneuve-ia-Garenne, France), and expression was determined by indirect fluorescent-antibody staining with a monoclonal antibody to the JE virus E protein (J3-11B9) (24). When transfection protocols recommended by the manufacturer were used, E antigen could be detected with this antibody in 3 to 5% of either COS7 or Vero cells 1 to 2 days after transfection, indicating that pcDNA3JEME expressed the E antigen in these eukaryotic cells.

Induction of NEUT antibodies and protective immunity. ICR mice were inoculated i.m. or i.d. with 10 or 100 μg of pcDNA3JEME at 4 and 6 weeks of age (Table 1). At 2 weeks after each immunization (at 6 and 8 weeks of age), sera were collected and checked for NEUT antibodies. Following the second serum collection, these mice were challenged with 10,000 LD_{50} of the P3 strain of JE virus and observed for 3 weeks; sera were collected at the end of the 3-week observation period from all mice that survived the challenge (at 11 weeks of age). The results in Table 1 show that one immunization with pcDNA3JEME did not induce detectable levels of NEUT antibodies but that two immunizations induced NEUT antibodies at titers of 1:10 to 1:20, irrespective of the immunization route and the dose. All mice given two inoculations survived the challenge but displayed significantly increased serum NEUT titers (1:160 to 1:640) at 3 weeks postchallenge. Mice inoculated with PBS did not have detectable levels of NEUT antibodies and died from the challenge, whereas mice vaccinated by inoculation with the Nakayama strain of JE virus 4 weeks prior to challenge had a high NEUT antibody titer and survived the P3 virus challenge. Since three of five mice died following infection with 5×10^6 PFU of the Nakayama strain of JE virus, this "vaccinating" dose corresponded to approximately 1 LD_{50}. These results indicate that two immunizations with pcDNA3JEME induced NEUT antibodies and protective immunity in mice. Since a significant difference in NEUT antibody titer and survival rate was not observed between the i.m. and i.d. routes, we used the i.m. route for subsequent experiments.

Next, the ability of pcDNA3JEME to induce protective im-

TABLE 2. Immunogenicity of pcDNA3JEME in ICR mice with one immunization

Immunogen[a]	Route	Dose (μg) at 4 wk[b]	NEUT titer[c] at wk[b]		Survival[d]
			7	10	
pcDNA3JEME	i.m.	100	<1:10	1:160	4/5
pcDNA3JEME	i.m.	10	<1:10	NA[e]	0/5
pcDNA3JEME	i.m.	1	<1:10	NA	0/5
pcDNA3JEME	i.m.	0.1	<1:10	NA	0/5
pcDNA3	i.m.	100	<1:10	NA	0/5
PBS	i.m.		<1:10	NA	0/5

[a] Each immunogen was given to five female ICR mice at 4 weeks of age, and the mice were challenged at week 7.
[b] Ages of the mice at the time of inoculations or serum collections; 10 weeks indicates 3 weeks postchallenge.
[c] Represented as the serum dilution yielding a 90% reduction in plaque number.
[d] Number of surviving mice/total number of mice tested 3 weeks after challenge with 10,000 LD_{50} of the P3 strain of JE virus.
[e] NA, serum was not available.

munity in ICR mice was examined in the one-immunization protocol at 0.1 to 100 μg (Table 2). Immunization was performed at 4 weeks of age, with bleeding and challenge at 7 weeks, observation for 3 weeks, and postchallenge bleeding when the mice were 10 weeks old. Nonimmune control groups included mice inoculated with PBS or pcDNA3 at 100 μg. All mice immunized with pcDNA3JEME at 0.1 to 10 μg, pcDNA3, and PBS died from challenge, but partial protection was observed for mice immunized with pcDNA3JEME at 100 μg. NEUT antibodies were not observed for prechallenge sera for any of these groups, but a high NEUT antibody titer was observed for surviving mice immunized with 100 μg of pcDNA3JEME.

Induction of specific CTLs. In order to study cellular responses in mice immunized with pcDNA3JEME, spleen cells were obtained from BALB/c mice immunized with pcDNA3JEME at 100 μg twice at a 2-week interval, stimulated in vitro with JE virus, and examined for cytotoxic activity against PMK cells infected with JE virus. Figure 1 shows the results obtained at an E/T ratio of 100:1. A high percentage of specific lysis of JE virus-infected cells (approximately 50%) was obtained with effector cells stimulated with JE virus but not with unstimulated effector cells. Cytotoxic activities against mock-infected cells were low. This result indicates that immunization with pcDNA3JEME induced JE virus-specific memory CTLs in mice.

The ability of pcDNA3JEME to induce specific CTLs in BALB/c mice was examined in the one-immunization protocol

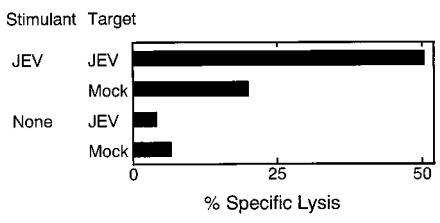

FIG. 1. Lysis of JE virus-infected PMK cells by pcDNA3JEME-immune spleen cells stimulated with JE virus. BALB/c mice were immunized with pcDNA3JEME at 100 μg twice at an interval of 2 weeks. At 2 weeks after the second immunization, the spleen cells were harvested and stimulated by incubation with C6/36-grown virus (JEV) or culture fluid from uninfected C6/36 cells (None) for 6 days. Cytotoxic activities against JE virus-infected (JEV) or mock-infected (Mock) PMK cells were measured at an E/T ratio of 100:1 by the standard chromium release method (see Materials and Methods for details).

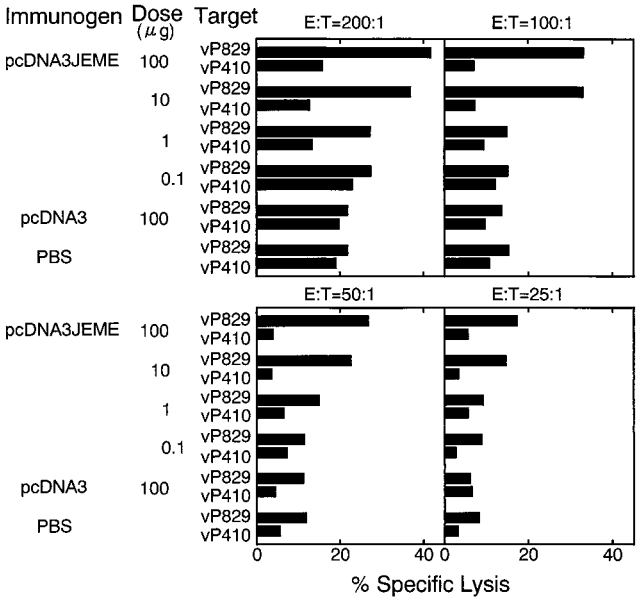

FIG. 2. Lysis of P815 cells infected with a recombinant vaccinia virus carrying the prM and E genes (vP829) or the parental virus (vP410) by pcDNA3JEME-immune spleen cells stimulated with JE virus. BALB/c mice were immunized once with pcDNA3JEME at 0.1 to 100 μg, pcDNA3 at 100 μg, or PBS. At 3 weeks postimmunization, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. Cytotoxic activities were measured at the indicated E/T ratios by the standard chromium release method (see Materials and Methods for details).

at 0.1 to 100 μg (Fig. 2). Specific cytotoxic activities were observed for mice immunized with 10 or 100 μg of pcDNA3JEME. However, responses in mice immunized with 1 or 0.1 μg of this DNA were not significantly different from the responses detected in samples collected from mice inoculated with 100 μg of pcDNA3 or PBS.

Characterization of CTLs. The phenotype of cells responsible for cytotoxic activities was determined by use of cell depletion tests (Fig. 3). Spleen cells obtained from BALB/c mice immunized twice with 100 μg of pcDNA3JEME and stimulated in vitro with JE virus were treated with antibodies against cell surface markers and complement before cytotoxicity assays. Cytotoxic activities were reduced by treatment with anti-CD3 or anti-CD8 in the presence of complement, whereas treatment with complement alone or with anti-CD4 and complement did not reduce the activities. These results indicate that

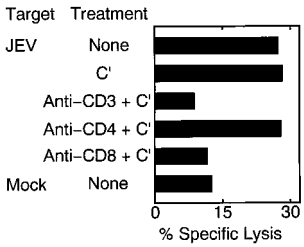

FIG. 3. Phenotypic analysis of CTLs by cell depletion with the indicated antibodies and complement. BALB/c mice were immunized with pcDNA3JEME at 100 μg twice at an interval of 2 weeks. Four weeks later, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. After cell depletion (see Materials and Methods for details), cytotoxic activities were measured at an E/T ratio of 120:1 with ^{51}Cr-labeled P815 cells infected with vP829. JEV, JE virus; Mock, mock infected.

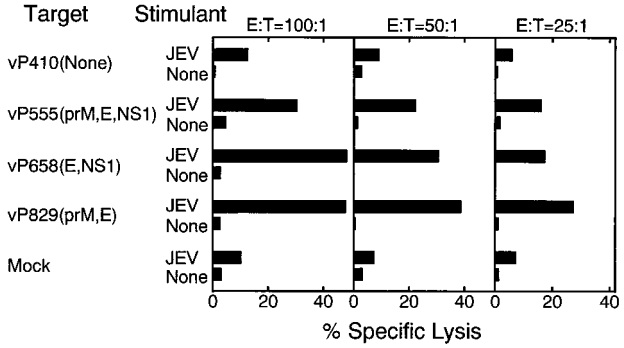

FIG. 4. Target antigen analysis of CTLs. BALB/c mice were immunized with pcDNA3JEME at 100 μg twice at an interval of 2 weeks. At 3 weeks later, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. Cytotoxic activities were measured at the indicated E/T ratios with ^{51}Cr-labeled P815 cells infected with a recombinant vaccinia virus encoding no antigens (parental virus vP410), prM, E, and NS1 (vP555), E and NS1 (vP658), or prM and E (vP829) or mock infected (Mock). JEV, JE virus.

CD8-positive and CD4-negative T lymphocytes were responsible for cytotoxic activities.

We investigated JE virus proteins recognized by CTLs by using P815 cells infected with recombinant vaccinia viruses expressing different JE virus antigens (Fig. 4). Spleen cells obtained from BALB/c mice immunized twice with 100 μg of pcDNA3JEME and stimulated in vitro with JE virus were used for this experiment. Specific cytotoxic activities were observed against target cells infected with vP555, vP658, and vP829; all of these recombinant viruses expressed E protein with or without prM protein. These results suggest that the predominant CTLs induced in pcDNA3JEME-immunized mice recognized E protein. Since target cells expressing prM protein alone were not used in this experiment, the presence or absence of a minor population of CTLs which specifically recognize prM protein could not be determined.

Duration of immunity induced by pcDNA3JEME. The levels of NEUT antibodies and protective immunity were examined with groups of two male BALB/c mice at 1 to 6 months after one immunization with pcDNA3JEME at 100 μg. Since undetectable levels of NEUT antibodies were found in ICR mice 3 weeks after one immunization (at 7 weeks of age; Table 2), the BALB/c mice in this experiment testing duration of immunity were challenged at different intervals following immunization, and the induction of NEUT antibodies at 4, 8, and 21 days after challenge was used as an indicator of the presence of vaccination-induced memory B cells (Table 3). As expected, prior to challenge (day −2 in Table 3), only low or undetectable NEUT antibody titers were observed for each pair of mice immunized with pcDNA3JEME, and no NEUT antibodies were detectable in mice inoculated with PBS. Following challenge, the NEUT antibody titers in mice immunized with pcDNA3JEME were elevated to 1:160 to 1:640 by day 4 and to 1:320 to 1:1,280 by day 8, and these levels were maintained or further increased until day 21. On the other hand, the titers of NEUT antibodies in unimmunized mice remained low (undetectable or 1:10) until days 4 and 8. All unimmunized mice died before day 10, and all immunized mice survived throughout the observation period (21 days). Although only two mice were used per group in this experiment, the results indicate that mice immunized with pcDNA3JEME maintained sufficient memory B cells to supply high titers of NEUT antibodies if challenged within 6 months of immunization.

The levels of memory CTLs were examined with two male BALB/c mice at 6 months after one immunization with pcDNA3JEME at 100 μg. The percentages of specific lysis obtained against vP829- and vP410-infected targets were 39.3 and 13.6% at an E/T ratio of 400:1 and 23.9 and 5.4% at an E/T ratio of 200:1, respectively. This result indicates that mice immunized with pcDNA3JEME maintained detectable levels of memory CTLs for at least 6 months.

DISCUSSION

This paper demonstrates that the JE virus prM and E genes introduced into mice in the form of plasmid DNA induced NEUT antibodies, CTLs, and protective immunity. In flavivirus infections, the prM, E, and NS1 proteins have been considered to induce protective immunity, since protection of mice from lethal challenge has been shown by passive transfer of monoclonal antibodies against the prM (10), E (11, 24) and NS1 (7) proteins. Recently, several epitopes on other viral proteins important for the induction of cellular immunity, including CTLs, were analyzed (5, 20, 21), but no epitopes were demonstrated to be responsible for protection. Although passive transfer of JE virus-specific CTLs protected mice from lethal challenge (27), the epitopes recognized by these CTLs were not characterized.

Our previous studies with poxvirus-based recombinant JE viruses demonstrated the proper synthesis of intracellular and extracellular forms of prM and E proteins in cells infected with recombinants encoding the signal sequence of prM, prM, and E, independent of the vector virus used: vaccinia virus (15, 16), canary poxvirus (18), and Sindbis virus (30). Furthermore, a similar cassette can be used to synthesize extracellular particles containing the structural proteins of other flaviviruses, including yellow fever virus (29), dengue type 1 virus (4), and tick-borne encephalitis virus (1). In the present study, we showed that a plasmid carrying these JE virus genes could be used to produce E protein in COS7 and Vero cells. Since the in vitro transfection efficiency of pcDNA3JEME was low in these cells, we did not attempt to identify extracellular forms of E in transfected cell cultures. However, the proper synthesis of E in the transfected cells was supported by the induction of specific

TABLE 3. Duration of immunity to pcDNA3JEME in BALB/c mice[a]

Interval between immunization and challenge (mo)[b]	Immunogen	NEUT titer[c] at day[d]				Survival[e]
		−2	4	8	21	
1	pcDNA3JEME	1:10	1:160	1:320	1:640	2/2
1	PBS	<1:10	1:10	1:10	NA[f]	0/2
2	pcDNA3JEME	<1:10	1:160	1:640	1:640	2/2
4	pcDNA3JEME	1:10	1:160	1:640	1:640	2/2
4	PBS	<1:10	1:10	NA	NA	0/2
6	pcDNA3JEME	1:10	1:640	1:1,280	≥1:1,280	2/2

[a] The duration was demonstrated by protection from challenge and anamnestic antibody response at up to 6 months following a single immunization.

[b] Groups of two male BALB/c mice were inoculated with PBS or 100 μg of pcDNA3JEME at 6 weeks of age and then challenged 1, 2, 4, or 6 months later.

[c] Represented as the serum dilution yielding a 90% reduction in plaque number.

[d] Days relative to challenge. Day −2 indicates 2 days before challenge.

[e] Number of surviving mice/total number of mice tested 21 days after challenge with the same dose of the P3 strain of JE virus as was used for the challenge of ICR mice (Table 2).

[f] NA, serum was not available due to the death of mice. Two mice immunized with PBS 1 month earlier died on days 6 and 10, and two mice immunized with PBS 4 months earlier died on days 6 and 8.

antibodies, CTLs, and protective immunity in mice inoculated with pcDNA3JEME.

Several devices to increase the level of expression of JE virus-proteins were incorporated into pcDNA3JEME. We chose a vector with a strong eukaryotic promoter derived from human cytomegalovirus and a well-characterized polyadenylation signal derived from bovine growth hormone. We inserted a strong eukaryotic initiation site containing an ACC sequence which precedes the AUG start codon and which has been reported to be an optimal sequence for initiation by eukaryotic ribosomes (19). We also altered the prM signal sequence to enhance expression. Specifically, we added 5 amino acids to the 15-amino-acid sequence that we used in earlier poxvirus recombinants (15, 25), based on results which were obtained with recombinant vaccinia viruses encoding similar cassettes for other flavivirus genomes and which showed that longer prM signal sequences resulted in higher levels of synthesis of extracellular particles (data not shown). Furthermore, we did not include the gene for NS1 in pcDNA3JEME, since the production of extracellular particles (which we believe are the critical immunogens) from cells infected with vP829 carrying the prM and E genes was eight times higher than the production with vP555 carrying the prM, E, and NS1 genes and since vP829 induced higher levels of protective immunity than vP555 in mice (15).

Induction of CTLs is one of the prominent features of DNA immunization. Cumulative experimental data have established a theory that peptides expressed by foreign genes introduced into cells are bound to major histocompatibility complex class I molecules and are recognized by CD8-positive T lymphocytes, including CTLs (6). In the present study, memory CTLs were demonstrated in pcDNA3JEME-immunized BALB/c mice. CTLs induced by pcDNA3JEME immunization recognized mainly E protein, consistent with our previous data indicating that recombinant poxviruses carrying prM, E, and NS1 proteins induced CTLs that recognized mainly E protein (13). Interestingly, mice immunized once with 100 μg of pcDNA3JEME, which did not induce high levels of NEUT antibodies, were protected from lethal challenge. Immunization with the same dose of pcDNA3JEME induced CTLs in BALB/c mice, making it tempting to speculate that the protection observed in animals given a single dose of 100 μg was due to CTL responses. However, it is possible that low levels of antibodies (below the detection limit in our assay) present prior to challenge or antibodies produced by memory B cells and helper T cells that were rapidly activated following i.p. exposure to the challenge virus may have been responsible for protection. Current studies are aimed at determining the components that confer protection in our murine challenge system.

Consistent with the current view on protection from cytopathic viruses (9), studies of JE virus suggest that preexisting antibodies provide the critical and predictive factor in protection. In our previous experiments, recombinant vaccinia viruses that express the E protein synthesized in a misfolded form in infected cells failed to induce NEUT antibodies and provided little protection from challenge (25). In the present study, using DNA vaccines, we discovered an immunization strategy in which animals with low or undetectable levels of NEUT antibodies were protected from challenge. Following challenge, the sera from these animals contained high levels of NEUT antibodies, indicating a significant secondary immune response due to the challenge virus (probably due to replication at peripheral sites). We previously reported the replication of challenge virus in mice which had high prechallenge NEUT titers against JE virus (15), indicating that sterile immunity may be difficult to achieve in our challenge system. Nevertheless, the data presented in this paper suggest that protection induced by delivery of JE virus gene subsets by recombinant viruses or as naked DNA may result from a different mechanism. Thus, in addition to its usefulness as a vaccine candidate, naked DNA immunization could be useful for elucidating the mechanisms of protection against flavivirus diseases.

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REFERENCES

1. **Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J. Virol. **69:**5816–5820.
2. **Cooney, E. L., A. C. Collier, P. D. Greenberg, R. W. Coombs, J. Zarling, D. E. Argitti, M. C. Hoffman, S. L. Hu, and L. Corey.** 1991. Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. Lancet **337:**567–572.
3. **Donnelly, J. J., J. B. Ulmer, and M. A. Liu.** 1994. Immunization with DNA. J. Immunol. Methods **176:**145–152.
4. **Fonseca, B. A. L., S. Pincus, R. E. Shope, E. Paoletti, and P. W. Mason.** 1994. Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice. Vaccine **12:**279–285.
5. **Gagnon, S. J., W. Zeng, I. Kurane, and F. A. Ennis.** 1996. Identification of two epitopes on the dengue 4 virus capsid protein recognized by a serotype-specific and a panel of serotype-cross-reactive human $CD4^+$ cytotoxic T-lymphocyte clones. J. Virol. **70:**141–147.
6. **Germain, R. N.** 1993. Antigen processing and presentation, p. 629–676. *In* W. E. Paul (ed.), Fundamental immunology, 3rd ed. Raven Press Ltd., New York, N.Y.
7. **Henchal, E. A., L. S. Henchal, and J. J. Schlesinger.** 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J. Gen. Virol. **69:**2101–2107.
8. **Johnson, M. P., C. A. Meitin, B. S. Bender, and P. A. Small, Jr.** 1988. Passive immune serum inhibits antibody response to recombinant vaccinia virus, p. 189–192. *In* H. Ginsberg, F. Brown, R. A. Lerner, and R. M. Chanock (ed.), Vaccines 88. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Kagi, D., and H. Hengartner.** 1996. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr. Opin. Immunol. **8:**472–477.
10. **Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels.** 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hyg. **41:**576–580.
11. **Kimura-Kuroda, J., and K. Yasui.** 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. J. Immunol. **141:**3606–3610.
12. **Kochel, T., S. J. Wu, K. Raviprakash, P. Hobart, S. Hoffman, K. Porter, and C. Hayes.** 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicits neutralizing antibodies in mice. Vaccine **15:**547–552.
13. **Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, and F. A. Ennis.** 1997. Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus-specific $CD8^+$ cytotoxic T lymphocytes in mice. Virology **227:**353–360.
14. **Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, N. Kanesa-Thasan, J. J. Smucny, C. H. Hoke, Jr., and F. A. Ennis.** Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Vaccine, in press.
15. **Konishi, E., S. Pincus, B. A. L. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason.** 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology **185:**401–410.
16. **Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason.** 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.
17. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714–720.
18. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, and P. W. Mason.** 1994. Avipox virus-vectored Japanese encephalitis virus vaccines: use as vaccine candidates in combination with purified subunit immunogens. Vaccine **12:** 633–638.

19. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:** 283–292.
20. **Kurane, I., Y. Okamoto, L. C. Dai, L. L. Zeng, M. A. Brinton, and F. A. Ennis.** 1995. Flavivirus-cross-reactive, HLA-DR15-restricted epitope on NS3 recognized by human $CD4^+$ $CD8^-$ cytotoxic T lymphocyte clones. J. Gen. Virol. **76:**2243–2249.
21. **Livingston, P. G., I. Kurane, L. C. Dai, Y. Okamoto, C. J. Lai, R. Men, S. Karaki, M. Takiguchi, and F. A. Ennis.** 1995. Dengue virus-specific, HLA-B35-restricted, human $CD8^+$ cytotoxic T lymphocyte (CTL) clones. Recognition of NS3 amino acids 500 to 508 by CTL clones of two different serotype specificities. J. Immunol. **154:**1287–1295.
22. **Manickan, E., K. L. Karem, and B. T. Rouse.** 1997. DNA vaccines—a modern gimmick or a boon to vaccinology? Crit. Rev. Immunol. **17:**139–154.
23. **Mason, P. W.** 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology **169:**354–364.
24. **Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, Jr., D. S. Burke, M. J. Fournier, and T. L. Mason.** 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J. Gen. Virol. **70:**2037–2049.
25. **Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti.** 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.
26. **McAda, P. C., P. W. Mason, C. S. Schmaljohn, J. M. Dalrymple, T. L. Mason, and M. J. Fournier.** 1987. Partial nucleotide sequence of the Japanese encephalitis virus genome. Virology **158:**348–360.
27. **Murali-Krishna, K., V. Ravi, and R. Manjunath.** 1996. Protection of adult but not newborn mice against lethal intracerebral challenge with Japanese encephalitis virus by adoptively transferred virus-specific cytotoxic T lymphocytes: requirement for $L3T4^+$ T cells. J. Gen. Virol. **77:**705–714.
28. **Phillpotts, R. J., K. Venugopal, and T. Brooks.** 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. **141:**743–749.
29. **Pincus, S., P. W. Mason, E. Konishi, B. A. L. Fonseca, R. E. Shope, C. M. Rice, and E. Paoletti.** 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.
30. **Pugachev, K. V., P. W. Mason, and T. K. Frey.** 1995. Sindbis vectors suppress secretion of subviral particles of Japanese encephalitis virus from mammalian cells infected with SIN-JEV recombinants. Virology **209:**155–166.
31. **Robinson, H. L., L. A. Hunt, and R. G. Webster.** 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin expressing plasmid DNA. Vaccine **11:**957–960.
32. **Rolph, M. S., and I. A. Ramshaw.** 1997. Recombinant viruses as vaccines and immunological tools. Curr. Opin. Immunol. **9:**517–524.
33. **Rooney, J. F., C. Wohlenberg, K. J. Cremer, B. Moss, and A. L. Notkins.** 1988. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. J. Virol. **62:**1530–1534.
34. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, and A. Friedman.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
35. **Wang, B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner.** 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **90:**4156–4160.
36. **Xiang, Z. Q., S. Spitalnik, M. Tran, W. H. Wunner, J. Cheng, and H. C. J. Ertl.** 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology **199:**132–140.