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Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus

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Abstract

We have constructed a series of plasmids encoding premembrane (prM) and envelope (E) protein genes of dengue virus type 2 (DEN-2). These plasmids included an authentic DEN-2 prM-E construct (pCBD2-14-6), and two chimeric constructs, 90% DEN-2 E-10% Japanese encephalitis (JE) virus E (pCB9D2-1J-4-3) and 80% DEN-2 E-20% JE E (pCB8D2-2J-2-9-1). Monoclonal antibody (MAb) reactivity indicated that all three plasmids expressed authentic DEN-2 virus E protein epitopes representative of flavivirus domains 1, 2, and 3. However, only the pCB8D2-2J-2-9-1 construct secreted high levels of prM, M (membrane), and E proteins into the culture fluid of plasmid-transformed COS-1 cells. The major portion of the prM and E proteins expressed by COS-1 cells transformed by pCBD2-14-6 or pCB9D2-4-3 plasmids remained membrane-bound. The results supported the notion that an unidentified membrane retention sequence is located between E-397 and E-436 of DEN-2 virus E protein. Replacing the carboxyl-terminal 20% of DEN-2 E (397-450) with the corresponding JE sequence had no effect on anti-DEN-2 MAb reactivity, indicating that this region is antigenically inert, although it is required for antigen secretion. Plasmid pCBD2-2J-2-9-1, which expressed secreted forms of prM/M and E that have the potential to form subviral particles, was superior to other constructs in stimulating an antibody response. Ninety percent neutralization titers ranging from 1:40 to >1:1000 were observed in seven of nine serum specimens from pCB8D2-2J-2-9-1-immunized mice. Eleven of twelve 2-day-old neonatal mice, derived from a pCB8D2-2J-2-9-1 immunized female mouse, survived intraperitoneal challenge of DEN-2 New Guinea C virus.

Keywords: Dengue virus type 2; Japanese encephalitis virus; Chimeric plasmid; Premembrane and envelope proteins; Neutralizing antibody

Introduction

[1]Dengue (DEN) fever, an acute infection that occurs in subtropical and tropical areas, is the most important arthropod-borne viral disease of humans (Monath, 1994). DEN viruses belong to the family of *Flaviviridae*, genus *flavivirus*. There are four distinct DEN virus serotypes: DEN-1, -2, -3, and -4. Infection with any of these viruses may be asymptomatic, or cause a self-limited febrile illness known as dengue fever (DF). In a small percentage of cases, however, infection results in a life-threatening dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). There are approximately 100 million cases of DF occurring annually worldwide and an estimated 500,000 hospitalized DHF/DSS reported. A safe and effective DEN vaccine is essential to provide protection for all four virus serotypes in DEN endemic and epidemic regions of the world. A live-attenuated DEN vaccine has shown promising results in phase 1 clinical trials (Bhamarapravati and Sutee, 2000; Kanesathasan et al., 2001). The reverse genetic technique using infectious cDNA clones derived from yellow fever (YF)

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vaccine virus (17D), attenuated DEN-2 vaccine virus (PDK-53), and genetically engineered, attenuated DEN-4 (814669) has greatly increased the potential of creating a genetically engineered, live-attenuated chimeric DEN vaccine (Blaney et al., 2001; Guirakhoo et al., 2000; Huang et al., 2000).

The use of DNA-based vaccines is a novel and promising immunization approach for the development of flavivirus vaccines (Chang et al., 2001). Few of the flavivirus DNA vaccines have been evaluated in the experimental animals other than mice. Candidate DNA vaccine for Russian spring-summer encephalitis, Central European encephalitis, and DEN-1 viruses have been evaluated in nonhuman primates (Kochel et al., 2000; Raviprakash et al., 2000; Schmaljohn et al., 1999). Japanese encephalitis (JE) virus DNA vaccine was evaluated in swine (Konishi et al. 2000b). Recently, we reported the development of highly immunogenic, recombinant DNA vaccines for JE and West Nile (WN) viruses that induced protective immunity in mice following a single intramuscular (im) injection (Chang et al., 2000; Davis et al., 2001). The recombinant WN virus construct also induced protective immunity in horses following a single im injection (Davis et al., 2001).

The protective immunity induced after DNA vaccination may correlate with the ability of plasmid DNA to direct synthesis of secreted premembrane/membrane (prM/M) and envelope (E) proteins by the host cells. COS-1 cells transformed with tick-borne encephalitis (TBE), JE or WN plasmids secreted the TBE, JE or WN prM/M and E proteins in the form of extracellular subviral particles (EPs) into the culture fluid (Allison et al., 1999; Davis et al., 2001; Hunt et al., 2001). The highly immunogenic, noninfectious EPs have potential not only as biosynthetic subunit vaccines but also as serodiagnostic antigens to replace virus-infected suckling mice brain preparations (Davis et al., 2001; Hunt et al., 2001; Konishi and Fujii, 2002; Konishi et al., 1997, 2001). In the present study, we have identified a potential membrane retention region between E-397 and E-436 of DEN-2 virus E protein. Replacing DEN-2 virus E-436 to E-450 with the corresponding E region of JE virus to construct a chimeric plasmid containing 90% DEN-2 E and 10% JE E gene had only minor effect on the prM and E secretion. However, the DEN-2-JE virus chimeric plasmid, consisting of the transmembrane signal sequence of JE virus, followed by prM and 80% E (E-1 to 397) of DEN-2, and 20% E of JE virus (corresponding to DEN-2 E-397 to E-450), secreted prM/M and E proteins efficiently in plasmid transformed COS-1 cells. The immune response in mice after im immunization of DEN-2 plasmids correlated with the efficiency of prM/M and E secretion. Only the construct efficiently secreting prM/M and E antigens had the capacity to stimulate high-titered neutralizing (Nt) antibodies in plasmid-vaccinated mice.

Results

Transient expression of DEN-2 virus recombinant antigen

Expression of the prM and E genes of DEN-2 virus or a chimeric E gene from a combination of DEN-2 and JE virus sequences (80% DEN-20% JE or 90% DEN-10% JE) was accomplished by separate transformations of each of the three recombinant DEN-2 DNA plasmids into COS-1 cells. The basic plasmid design was based on results from previous studies with JE virus and WN virus recombinant plasmids in which plasmid-transformed cells expressed and secreted authentic viral proteins into the cell culture fluid (Chang et al., 2000; Davis et al., 2001). Transient expression of DEN-2 recombinant proteins was initially assessed by Ag-capture ELISA of cell-culture supernatants and by immunofluorescence antibody assay (IFA) of acetone-fixed, transformed COS-1 cells (Chang et al., 2000). The optimum antigen expression was determined to be 48 h following electroporation.

Epitope mapping of the E protein expressed by transiently transformed COS-1 cells

The DEN-2 protein expressed by each of the recombinant plasmids was evaluated by IFA using a panel of murine monoclonal antibodies (MAbs) with known reactivity to DEN-2 virus (Table 1) (Henchal et al., 1985; Roehrig et al., 1998). The MAb panel included antibodies reactive with each of the three antigenic domains of the E protein of flaviviruses as well as prM and C proteins (Guirakhoo et al., 1989; Rey et al., 1995). The MAbs specific for flavivirus antigenic Domains 2 and 3 showed nearly identical qualitative reactivity with DEN-2 virus and each of the three plasmid-expressed proteins. Domain 1 specific MAb 1B4C-2 also showed a similar reactivity pattern with all expressed proteins. However, two of the Domain 1 specific MAbs, 2B3A-1 and 9A4D-1, were much less reactive with E protein expressed by plasmids pCBD2-14-6 and pCB9D2-1J-4-3 as shown by endpoint titration (values in parentheses, Table 1). Comparison of the endpoint titers revealed the apparent poor expression of epitopes C3 and C4 in constructs containing 100% DEN-2 E and 90% DEN-2 E-10% JE E. MAb 2H2, specific for prM, had the same reactivity with antigen expressed by all three plasmids. Anti-C MAb 1A2A-1 reacted well with DEN-2 virus and had low-level, nonspecific reactivity with the plasmid-expressed viral proteins, which included prM and E, but not C.

Comparison of secreted protein and membrane-bound protein produced by each of the three DEN-2 recombinant plasmids

Similar amounts of cell-culture fluid were harvested from COS-1 cells 48 h posttransformation for each recombinant DEN-2 plasmid. The DEN-2 viral antigens secreted

Table 1
Characterization of DEN-2 E glycoprotein epitopes expressed by the recombinant DEN-2 plasmids as determined by the indirect immunofluorescence antibody assay (IFA)

Antibody			Controls[a]		Plasmid construct[a]		
MAb (epitope)[b]	Antigenic domain[c]	PRNT[d]	DEN-2-infected cells	Normal cells	pCBD2-14-6 (100D2-E)	pCB9D2-IJ-4-3 (90D2-E-10JE-E)	pCB8D2-2J-2-9-1 (80D2-E-20JE-E)
4G2	2	+/−	4+	—	4+	4+	4+
6B6C-1 (A1)	2	+/−	4+	—	4+	4+	3–4+
4E5 (A2)	2	Yes	3+	—	3–4+	3–4+	2–3+
1B7 (A5)	2	Yes	3–4+	—	4+	4+	2–3+
1B4C-2(C1)	1	No	3–4+ (8000)	—	2–3+ (4000)	2–3+ (4000)	2–3+ (8000)
2B3A-1 (C3)	1	No	3–4+ (≥3200)	—	3+ (100)	2+ (100)	2–3+ (≥3200)
9A4D-1 (C4)	1	No	3–4+	—	2–3+ (400)	1–3+ (400)	3+ (≥12800)
3H5 (B2)	3	Yes	4+	—	4+	4+	4+
10A4D-2 (B3)	3	Yes	2–3+	—	3–4+	3–4+	2–3+
1AID-2 (B4)	3	Yes	4+	—	3–4+	4+	3–4+
9D12-6	?	Yes	2–4+		2–3+	2–3+	3–4+
2H2	prM	No	4+	—	4+	3–4+	3–4+
1A2A-1	Capsid	No	2–3+	—	1+	2+	1–2+

[a] IFA substrates were acetone-fixed COS-1 cells, infected with DEN-2 16681, uninfected controls, or transformed with a DEN-2 recombinant plasmid.
[b] Monoclonal antibodies were used at a predetermined optimum dilution based on reactivity with DEN-2 16681 virus. For some MAbs, endpoint titers, shown in parentheses, are reported and for others, only qualitative values are reported based on a scale from 1+ to 4+, with 3–4+ considered positive, 2+ equivocal, and 1+ negative.
[c] Antigenic domains based on the E-glycoprotein of TBE virus (12, 30).
[d] Plaque-reduction neutralization activity at a 1:100 dilution of ascitic fluid, using a 90% plaque-reduction endpoint, except for 4G2 and 9D12-6, for which a 50% neutralization endpoint is reported (14, 33).

into untreated culture supernatants were first assessed by Ag-capture ELISA (Table 2). The cells transformed with 100% DEN-2 E and 90% DEN-2 E-10% JE E plasmids did not secrete DEN-2 viral antigens in replicate transformation experiments, thus, were ELISA-negative in unconcentrated culture supernatants. However, cells transformed with the 80% DEN-2 E-20% JE E plasmid secreted DEN-2 viral antigens and demonstrated ELISA titers between 1:8 and 1:32 in different transformation experiments. To increase the antigen concentration that may be undetectable by either Ag-capture ELISA or Western blot, an equivalent volume of the culture fluid from each plasmid was concentrated 100-fold by polyethylene glycol (PEG) precipitation, followed by ethanol extraction to remove PEG, which interfered with subsequent analysis by polyacrylamide gel electrophoresis. The relative amount of secreted antigen expressed by each plasmid was determined by Ag-capture ELISA analysis of both PEG-precipitated and ethanol-extracted cell-culture

Table 2
Detection of secreted and membrane-bound DEN-2 recombinant protein by antigen-capture ELISA

Plasmid	Sample type	Endpoint ELISA titer
pCBD2-14-6 (100D2-E)	Culture fluid	<1:1
	PEG-precipitated culture fluid[a]	<1:10
	PEG-precipitated, ethanol-extracted culture fluid[b]	<1:20
	Hydrophobic membrane protein preparation[c]	1:160
pCB9D2-1J-4-3 (90D2-E-10JE-E)	Culture fluid	<1:1
	PEG-precipitated culture fluid[a]	<1:10
	PEG-precipitated, ethanol-extracted culture fluid[b]	<1:20
	Hydrophobic membrane protein preparation[c]	1:80
pCB8D2-2J-2-9-1 (80D2-E-20JE-E)	Culture fluid	1:8–1:32
	PEG-precipitated culture fluid[a]	1:640
	PEG-precipitated, ethanol-extracted culture fluid[b]	1:80
	Hydrophobic membrane protein preparation[c]	1:80
pEGFP	Culture fluid	<1:1
	PEG-precipitated culture fluid[a]	<1:10
	PEG-precipitated, ethanol-extracted culture fluid[b]	<1:10
	Hydrophobic membrane protein preparation[c]	<1:10

[a] Culture supernatant from plasmid-transformed cells was precipitated with 10% polyethylene glycol (PEG) and resuspended in 1/100th of original volume.
[b] PEG-precipitated culture supernatant was extracted with 4% ethanol to remove PEG and the pellet was resuspended in 1/5 of the volume extracted.
[c] Hydrophobic membrane fractions were prepared as described under Materials and methods.

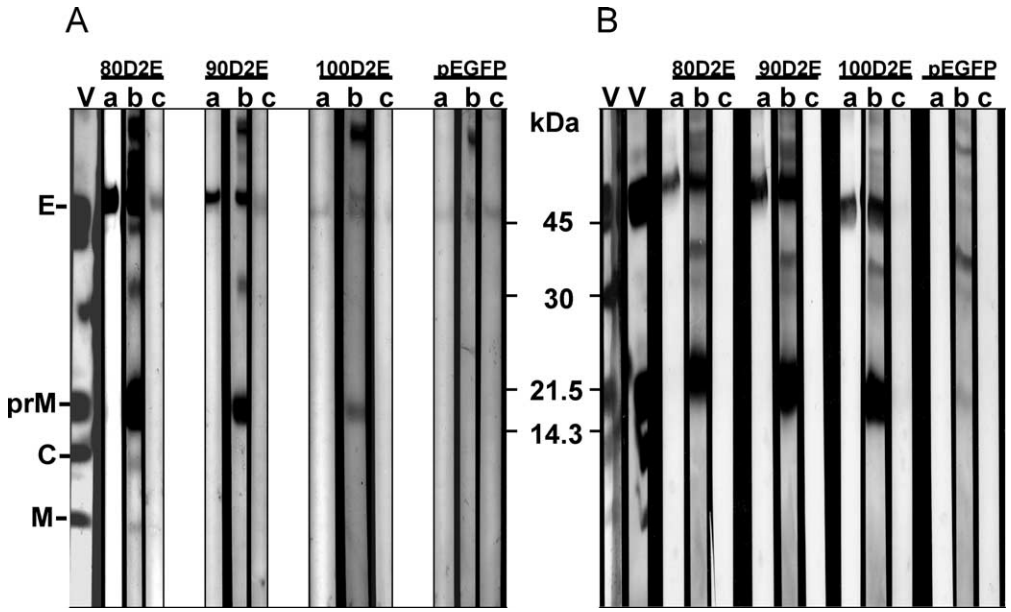

Fig. 1. Comparison of secreted and membrane-bound recombinant protein by Western blot. (A) Analysis of secreted recombinant antigen following PEG-precipitation and ethanol extraction of culture fluid for DEN-2 plasmids pCB8D2-2J-2-9-1 (80D2E), pCB9D2-1J-4-3 (90D2E), and pCBD2-14-6 (100D2E), and control plasmid pEGFP. Lane 1 (V), purified DEN-2 virus stained by Gold Blot (Owl Separation Systems, Portsmouth, NH). Reactivity of secreted, recombinant antigen from each plasmid with (a), anti-envelope (E) specific MAb 1A6A-8; (b), a mixture of MAb 1A6A-8, anticapsid (C) specific MAb 1A2A-1, antiserum specific for a DEN-2 virus membrane (M) protein peptide (M protein aa 1-34), and antiserum specific for DEN-2 virus premembrane (prM) protein; and (c), normal mouse ascites. (B) Analysis of recombinant plasmid-transformed cell hydrophobic membrane proteins. Lane 1 (V), purified DEN-2 virus stained by Gold Blot; Lane 2 (V), reactivity of purified DEN-2 virus with a mixture of MAb 1A6A-8, MAb 1A2A-1, antiserum specific for DEN-2 virus M protein, and antiserum for DEN-2 virus prM protein. Reactivity of isolated hydrophobic membrane proteins from each plasmid-transformed cell line with (a), MAb 1A6A-8; (b), a mixture of MAb 1A6A-8, MAb 1A2A-1, antiserum specific for DEN-2 virus M protein, and antiserum for DEN-2 virus prM protein; and (c), normal mouse ascites.

fluid preparations (Table 2). Secreted antigen was detected only from cells transformed with pCB8D2-2J-2-9-1, which contained 80% DEN-2 E and 20% JE E genes. The recombinant plasmids containing either 100% DEN-2 E or 90% DEN-2 E-10% JE E genes produced no ELISA-detectable antigen in the culture fluid, despite efforts to concentrate expressed protein.

Western blot analysis was also used to evaluate the production of secreted antigen by each of the DEN-2 recombinant plasmids. For comparison purposes, equivalent volumes of PEG-precipitated, ethanol-extracted cell-culture supernatant were run on NuPAGE gradient gels, electroblotted to nitrocellulose, and analyzed using MAbs or polyclonal antisera capable of reacting with all DEN-2 structural proteins (Fig. 1A). Western blot analysis showed greater sensitivity in detecting recombinant antigen than Ag-capture ELISA since DEN-2-specific proteins were detected in the culture fluid from two of the plasmids, pCB8D2-2J-2-9-1 and pCB9D2-1J-4-3. Plasmid pCB8D2-2J-2-9-1 expressed the greatest amount of secreted antigen which was shown to be composed of E, prM, and M proteins. The amount of M protein seemed to be variable for replicate transformations, perhaps due to varying efficiency of prM processing (data not shown). Relatively less secreted antigen was produced by pCB9D2-1J-4-3, and barely detectable levels were found for the pCBD2-14-6 preparation, which appeared to contain relatively less expressed E protein, especially if the nonspecific reactivity of the E-specific MAb, 1A6A-8, on the control pEGFP was taken into consideration (Fig. 1A, lanes a, b for 14-6 and GFP).

Since E, prM, and M are membrane-associated proteins throughout their intracellular synthesis, any assessment of the expression of these proteins by the three recombinant DEN-2 plasmids should include an evaluation of cell-membrane preparations from plasmid-transformed cells. The Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL) was used to isolate the integral membrane proteins from equivalent numbers of transformed cells for each recombinant plasmid. Hydrophobic proteins were separated from hydrophilic proteins by phase partitioning. Preliminary analysis by Ag-capture ELISA indicated that the hydrophilic protein fraction was nonreactive (data not shown); however, the hydrophobic protein fractions from COS-1 cells transformed with each of the recombinant DEN-2 plasmids had similar titers in ELISA tests (Table 2). These results indicated that recombinant antigen was expressed by all three plasmids following transformation, but was not always secreted.

Confirmation of the ELISA results for the hydrophobic

Table 3
Immunogenicity of three DEN-2 recombinant plasmids in ICR mice

Plasmid DNA[b]	Mouse No.	ELISA on DEN-2 virus					PRNT on DEN-2 virus[a] Endpoint titer 9 wks, p.v.	PRNT on JE virus[a] Endpoint titer 9 wks, p.v.
		Screen 3 wks, p.v.[c]		Screen 6 wks, p.v.[c]		Endpoint titer 9 wks, p.v.		
		1:100	1:400	1:100	1:400			
pCB8D2-2J-2-9-1	Pool, 1,2,4–10	ND[d]	ND	+	+	64,000	ND	ND
(80D2-E-20JE-E)	1	+	+	+	+	64,000	>1000	<2
	2	+	+	+	+	32,000	>1000	<2
	4	+	+	+	+	16,000	200	<2
	5	+	+	+	+	4000	<10	<2
	6	+	+	+	+	16,000	200	<2
	7	+	−	+	+	64,000	100	<2
	8	+	−	+	+	8000	40	<2
	9	+	+	+	+	6400	<2	<4
	10	+	+	+	+	64,000	>1000	<2
pCB9D2-IJ-4-3	Pool, 1–10	ND	ND	+	+	1000	ND	<2[e]
(90D2-E-10JE-E)	1	−	−	+	−	400	<10	ND
	2	+	−	+	+	200	<10	ND
	3	+	+	+	+	4000	<2	≤4
	4	+	−	+	−	200	<10	ND
	5	−	−	+	+	400	<10	ND
	6	+	+	+	+	4000	<2	2
	7	−	+/−	−	−	100	<10	ND
	8	−	−	−	−	200	<10	ND
	9	+	−	+	−	4000	<2	<2
	10	−	−	+	+	4000	<2	<2
pCBD2-14-6	Pool, 1–10	ND	ND	+	−	200	<2[f]	<2[g]
(100D2-E)	1	−	−	−	−	400	<10	ND
	2,3,6–9	−	−	−	−	<100	ND	ND
	4	+	+	+	+	1000	<2	<2
	5	−	−	+	−	2000	8	<2
	10	+	−	−	−	<100	ND	ND
PEGFP	Pool, 1–10	−	ND	−	ND	<100	<2	<2

[a] PRNT, plaque-reduction neutralization test, 90% neutralization endpoint.
[b] Mice were immunized intramuscularly with 100 μg plasmid DNA on weeks 0 and 3.
[c] ELISA screens used sera diluted 1:100 and 1:400.
[d] ND, not done.
[e] Pool, 1, 2, 4, 5, 7, 8.
[f] Pool, 2, 3, 6–10.
[g] Pool, 1–3, 6–10.

protein fractions was accomplished by Western blot (Fig. 1B). Equivalent volumes of hydrophobic protein fractions from each of the plasmid-transformed cells were diluted according to the manufacturer's recommendations for SDS–polyacrylamide gel electrophoresis to reduce band and lane distortion. Immunoblotting with E-, prM-, C-, and M-specific MAbs or polyclonal antisera showed that all three recombinant DEN-2 plasmids produced similar amounts of recombinant antigen composed of E and prM. No M protein was detected, either because it was not processed from prM or because the levels were too low to be detected. Despite efforts to reduce band distortion, high levels of detergent in the hydrophobic protein samples apparently caused E and prM to run in a slightly aberrant manner (slower migration) compared to samples without such high concentrations of detergents (compare E and prM migration in Figs. 1A and 1B).

Comparison of the immune response in mice vaccinated with three different DEN-2 recombinant DNA plasmids

To compare the extent and functionality of antibody responses stimulated by DNA vaccination, 3-week-old ICR mice were immunized by im injection with 100 μg of either pCB8D2-2J-2-9-1, pCB9D2-1J-4-3, pCBD2-14-6, or pEGFP on weeks 0 and 3. Outbred mice were selected for this study to provide the genetic heterogeneity expected in nonhuman primates planned for future study. Mice were bled 3, 6, and 9 weeks after the primary immunization. Individual and pooled sera were tested by indirect ELISA, using screening dilutions of 1:100 and 1:400 at 3 and 6 weeks postvaccination and endpoint titrations at 9 weeks postvaccination. Nine-week sera were also tested by plaque reduction neutralization test (PRNT) with both DEN-2 and JE viruses. The ELISA results showed that after one immu-

nization (3-week sera) all mice given pCB8D2-2J-2-9-1 had seroconverted, whereas only 50% of pCB9D2-1J-4-3- and 20% of pCBD2-14-6-vaccinated mice reacted with DEN-2 virus (Table 3). By 9 weeks postvaccination, all mice vaccinated with either pCB8D2-2J-2-9-1 or pCB9D2-1J-4-3 demonstrated anti-DEN-2 ELISA reactivity; however, the geometric mean titers were significantly different, 1:20,000 vs 1:708, respectively. Only 40% of pCBD2-14-6-immunized mice had anti-DEN-2 ELISA titers greater than 1:100. A Western blot of pooled 9-week sera from pCB8D2-2J-2-9-1-immunized mice on purified DEN-2 virus showed that the immunodominant response was to the E glycoprotein, with only slight reactivity detected to prM and M (data not shown).

An even more significant result in terms of evaluating the vaccine potential of the three DEN-2 plasmids was the induction of virus-Nt antibody in seven of nine mice immunized with pCB8D2-2J-2-9-1. The titers reported are based on a 90% plaque-reduction endpoint (Table 3); however, if a 70 or 50% neutralization endpoint is used, nine of nine sera have PRNT titers of $\geq$1:2 or $\geq$1:40, respectively (data not shown). Ninety percent neutralization titers ranged from 1:40 to >1:1000 for the seven sera with Nt activity. None of the mice immunized with pCB9D2–1J-4-3 produced Nt antibody, and only 1 of 10 sera from pCBD2-14-6-vaccinated mice neutralized virus, but at a titer of only 1:8. We did not challenge these vaccinated mice with DEN-2 virus since 12-week-old ICR mice are resistant to DEN-2 virus challenge by either intraperitoneal (ip) or intracranial route. For the purpose of comparison, mice challenged with DEN-2 New Guinea C strain by the ip route had a mean neutralization titer of 1:10 (Konishi et al., 2000a).

Since two of the recombinant plasmids contained JE virus E-gene sequences, all sera were evaluated for JE virus Nt activity; however, no activity was detected at the 90% neutralization endpoint for mice in any of the immunization groups. Mice immunized with the control plasmid pEGFP showed no reactivity to either DEN-2 or JE viruses.

Passive protection of neonatal mice derived from pCB8D2-2J-2-9-1-vaccinated female

Female ICR mice were vaccinated with one dose of 100 μg/100 μl of pCB8D2-2J-2-9-1 plasmid DNA at the age of 3 weeks. For evaluation of passive protection by maternal antibody, pups were obtained from a female that had Nt titer of 1:160 at 9-weeks postvaccination. Pups were challenged ip at 2 days after birth with 50,000 PFU/50 μl of NGC virus. Pups from an unvaccinated female, challenged at 1 day after birth with 50,000 PFU/50 μl of NGC virus, were used as unvaccinated controls. Eleven of 12 pups nursed by the vaccinated mother (PRNT titer of 1:160) survived viral infection, whereas none of 12 pups, receiving 50,000 PFU virus, from the control mother survived. Limited DEN-2 virus replication occurred in the survived pups since DEN-2 ELISA antibody (1:100) was detected in the serum of all survived pups at 25 days postchallenge.

Discussion

The use of plasmid vectors to express prM/M and E proteins that are able to stimulate a protective immune response is a novel and promising approach for the development of flavivirus vaccines (Chang et al., 2001). It is also an important step toward the development of a stably transformed cell line that constitutively produces and secretes noninfectious subviral particles as the potential source of a biosynthetic subunit vaccine and serodiagnostic antigen. Vaccination with the recombinant plasmid results in endogenous expression of prM and E proteins and stimulates anti-prM and E antibody responses in the host. We have designed a recombinant eukaryotic expression plasmid that contains optimized transcriptional and translational elements that enhanced the expression and secretion of JE and WN virus prM/M and E proteins into the culture medium of transiently transformed cells (Chang et al., 2000; Davis et al., 2001). Initially, the same strategy was applied to construct a recombinant DEN-2 plasmid, pCBD2-14-6, consisting of the authentic DEN-2 prM and E gene regions. Antigenic mapping of DEN-2 proteins expressed by the transformed COS-1 cells indicated that the prM and E proteins had a similar fluorescence intensity and MAb reactivity compared to virus-infected cells (Table 1). However, the staining pattern observed with plasmid-transformed cells was a globular pattern of intensely stained, large inclusion bodies as compared to the virus-infected cells, which showed a more evenly dispersed or diffused staining pattern.

Surprisingly, these pCBD2-14-6-transformed COS-1 cells failed to secrete a DEN-2 antigen detectable by Ag-capture ELISA, and plasmid vaccination failed to stimulate anti-DEN-2 virus Nt antibody in im immunized mice (Table 3). Studies with recombinant vaccinia viruses expressing C-terminally truncated E proteins suggested that the first 392 amino acids of the DEN-4 virus E sequence are critical in maintaining proper antigenic structure of the E protein and that the C-terminal 20% of DEN-4 E contains a membrane retention signal (Men et al. 1991). A similar membrane retention signal may also present in the C-terminal region of DEN-2 E and could have caused an intracellular accumulation of viral protein and a resultant pattern of globular fluorescent staining in the pCBD2-14-6-transformed, acetone-fixed cells. This IFA staining pattern was not observed in the JE or WN recombinant plasmid-transformed cells (Chang et al., 2000; Davis et al., 2001). We, therefore, constructed two additional plasmids, pCB9D2-1J-4-3 and pCB8D2-2J-2-9-1, for which 10 or 20% of the C-terminal DEN-2 E was replaced with the corresponding region of JE virus E protein. The pCB9D2-1J-4-3 plasmid secreted very low levels of prM and E proteins, detectable

only by Western blot in transiently transformed COS-1 cells; only pCB8D2-2J-2-9-1-transformed cells secreted DEN-2 virus prM/M and E proteins that could be detected both by Ag-ELISA and Western blot (Fig. 1 and Table 2).

Particle assembly and secretion is influenced by prM and E interaction. Studies on TBE virus have indicated that interactions between prM and the ectodomain of E are involved in prM-mediated intracellular transport of prM-E heterodimers. Interactions between prM and both a predicted stem region (H2) and a first transmembrane region (TM1) of E are factors stabilizing the prM-E interaction, presumably leading to particle formation (Allison et al., 1999). Replacement of the C-terminal 20% of DEN-2 E with the corresponding JE E sequence to construct pCB8D2-2J-2-9-1 resulted in DEN-2 prM and chimeric E secretion, most likely in a particulate form [based on the immunogenic quality of this antigen (Table 3)]. The major portion of the prM and E proteins expressed by pCBD2-14-6- and pCB9D2-4-3-transformed COS-1 cells remained membrane-bound (Table 2 and Fig. 1). This result substantiated the observation that a membrane retention sequence, similar to an unidentified sequence in DEN-4 virus E, is also present in the C-terminal stem region of the DEN-2 E protein. It also provided evidence that H2 and TM1 of JE E could interact with DEN-2 prM, perhaps stabilizing the prM-E interaction leading to prM/M and E protein secretion. The prM processing resulted in M protein-containing virion occurs at the latest stage during flavivirus maturation.

The prM protein is essential for maintaining proper conformation and secretion of the E protein during prM-E maturation (Aberle et al., 1999; Allison et al., 1995). For Murray Valley encephalitis virus, the ectodomain of E protein interacting with prM has been estimated to be between amino acids 200 and 327 (Guirakhoo et al., 1992). Recombinant vaccinia viruses expressing C-terminally truncated E proteins suggest that the first 392 amino acids of the DEN-4 virus E sequence is required for proper antigenic structure (Men et al., 1991). We believe that proper prM and E interactions as well as the structural integrity of E are maintained in the protein expressed by all three DEN-2 constructs. Replacement of the C-terminal 20% of E in pCB8D2-2J-2-9-1, conserving 397 amino acids of authentic DEN-2 E, was expected to have a minimum effect on the antigenic structure of E and to preserve prM-E interactions. Interactions of the prM and stem-anchor region of E are not virus-species specific. Replacing the stem-anchor region of DEN-2 E with the corresponding JE sequence had no effect on MAb reactivity (Table 1), indicating that this region is antigenically inert, but is required for prM/M and E secretion, and possibly assembly of prM/M-E containing EPs.

The plasmid construct responsible for secreting EPs of TBE virus prM and E protein was superior to constructs producing primarily intracellular or soluble forms of E protein in terms of extent and functionality of the antibody response (Aberle et al., 1999). Here we demonstrated that the ability of DEN-2 plasmid DNA to stimulate Nt antibody was correlated with the secretion of prM/M and E (Fig. 1, Tables 2 and 3). The morphology and physical character of secreted prM and E were not investigated in this study. It is likely that prM/M and E secreted by the pCB8D2-2J-2-9-1 construct forms EPs, which represent a potent immunogen with multiple, protective antigens presented on the particle surface.

Previous attempts to develop a DEN-2 virus DNA vaccine has resulted in various degrees of success (Kochel et al., 1997; Konishi et al., 2000a; Simmons et al., 2001). Vector components in those studies are similar to the present study, in which RNA transcripts of the DEN insert are regulated by the cytomegalovirus early gene promoter and bovine growth hormone poly(A) termination signal. However, the Nt-inducing efficiency of DNA vaccine in mice is significantly higher in our study than previous reports. Of the prM and E gene regions reported in the previous publications, one construct is similar to our 100% DEN-2 plasmid (Konishi et al., 2000a), and the other contains only 91% of the E gene (Kochel et al., 1997). Kozak's consensus sequence used in our construct could enhance the translation efficiency of the DEN-2 insert (Chang et al., 2000). The transmembrane signal peptide sequence (derived from JE virus) used in our construct has improved cleavage probability and could influence the proper translocation as well as processing of prM/M and E proteins. Signal peptide differences in various constructs may account for the difference in protein translocation, cleavage site presentation, and correct topology of prM and E proteins (Chang et al., 2001). However, it is highly probable that the higher Nt-inducing efficiency of the 80% DEN-2 E construct in mice in the present study is due to the synergistic effects of transcriptional and translational elements that increase both the secretability and the particle formation of DEN-2 prM and E proteins. Coimmunization of the immunostimulatory CpG motif-containing pUC19 plasmid, including plasmid-expressing murine GM-CSF in the vaccine regimen, or replacing the C-terminal 43 amino acids of E with lysosome-associated membrane retention sequence, improved the antibody response to the DEN-2 vaccine (Porter et al., 1998; Raviprakash et al., 2001).

Age-dependent resistance to viral infection in outbred mice has been observed in flaviviruses, including four serotypes of DEN, YF, St. Louis encephalitis, and JE viruses. The development of a consistent challenge protocol to address protective efficacy is essential for future multivalent DNA vaccine trials in mice. Previously, we observed that maternal antibodies could provide passive protection in neonatal mice from JE virus induced encephalitis by ip challenge (Chang et al., 2000). The protective efficacy in the baby correlated with the mother's serum Nt antibody titer. A similar strategy of immunizing female mice with the 80% DEN-2 E-20% JE E construct indicated that, indeed, maternal antibody capable of Nt DEN-2 virus could also provide passive protection in neonatal mice from DEN-2 virus ip challenge. Thus, enhancement of the vaccine potential of

Table 4
Oligonucleotides used to construct DEN-2 virus prM-E expression plasmids, and the junction region of chimeric DEN-2 and JE E indicated

100% DEN-2-prM-E:		
D2KasI-438[a]		5′ TGTGCA***GGCGCC***TTCCATTTAACCACACGTAACG
CD2NotI-2402		5′ TCGA***GCGGCCGC***TTCAACTAATTAGGCCTGCACCATGACTC
90% DEN-2-E & 10% JE-E:		
T7		5′CTTATCGAAATTAATACGACTCACTATAGG
CD2BstXI-2244		5′ATAGATTGCT***CCA***AAC*ACT****TGG***TGG
JE-2281		5′ ACTCCATAGGAAAAGCCGTTCACC
CSP6		5′ GCGAGCTCTAGCATTTAGGTGACACTATAG
	DEN-2←	**→JE**
90–10 Junction:	**Leu His Gln Val Phe Gly Gly Ala Phe Arg Thr**	
	CTC CAC CAA GTG TTT GGT GGT GCC TTC AGA ACA	
80% DEN-2-E & 20% JE-E:		
T7		5′ CTTATCGAAATTAATACGACTCACTATAGG
CD2BsmBI-2097		5′ GAATT***CGTCTC***ACTTCCTTTCTTAAACCAGTTGAGCTTC
JEBsmBI-2175		5′ GGAATT***CGTCTC***GGAAGCACGCTGGGCAAGG
CSP6		5′ GCGAGCTCTAGCATTTAGGTGACACTATAG 3′
	DEN-2←	**→JE**
80–20 Junction:	**Asn Trp Lys Lys Gly Ser Thr Leu Gly Lys Ala**	
	AAC TGG TTT AAG AAA GGA AGC ACG CTG GGC GCC	

[a] Restriction enzyme sites encoded in oligonucleotides were highlighted by bold, italics, and underlined functions.

flavivirus DNA can be achieved by manipulation of an expression plasmid for enhancing transcription and translation, promoting correct polyprotein processing and assembly, and targeting prM/M and E proteins for secretion.

We have constructed recombinant plasmids which secrete prM and E proteins for DEN-1 and DEN-3, similar to the 80% DEN-2 E construct in this study (G. J. Chang, unpublished results). We have also established DEN-1, -2, and -3 plasmid-transformed cell lines that constitutively express and secrete prM/M and E proteins for these three viruses. Objectives of an ongoing study are to obtain both DEN-4 and YF plasmid constructs and to establish DEN-4 and YF prM/M and E antigen-producing cell lines. Using candidate DNA vaccines for the four DEN serotypes, YF, JE, and WN viruses, a future study will address the vaccine potential of the formulated combination vaccine cocktails, based on the disease prevalence in different geographic regions. Noninfectious prM/M and E antigen-producing cell lines can provide biosynthetic subunit vaccines as well as standardized, noninfectious serodiagnostic antigens.

Materials and methods

Cell culture and virus strain

COS-1 cells (ATCC, Manassas, VA; 1650-CRL) were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's minimal essential medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 ml/l 7.5% $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin. Vero and C6/36 cells were grown under the same conditions used for COS-1 cells. DEN-2 virus strain-16681 was used for cDNA cloning, IgG ELISA, and the PRNT. Virus was propagated in C6/36 cell culture. Virus used for immunological or biochemical studies was purified by precipitation with 7% polyethylene glycol (PEG-8000; Fisher Scientific, Fair Lawn, NJ), followed by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (Obijeski et al., 1976).

Mouse brain adapted DEN-2 New Guinea C strain virus was used in the passive challenge experiment. This strain was propagated in suckling mouse by intracranial injection, and a 10% suspension of homogenized mouse brain was used for the challenge study.

Plasmid construction

Genomic RNA was extracted from 150 μl C6/36 cell-culture medium infected with DEN-2 (16681) using the QIAamp Viral RNA Kit (Qiagen, Santa Clarita, CA). Extracted RNA, resuspended in 80 μl DEPC (Sigma, St. Louis, MO), was used as a template in a reverse transcriptase-PCR (RT-PCR) for the amplification of DEN-2 virus prM/M and E genes. Primer sequences (Table 4) were designed based on the published sequence (Kinney et al., 1997). Restriction enzyme site *Kas*I was incorporated at the 5′-terminus of the cDNA amplicon. An in-frame termination codon, followed by a *Not*I restriction site, was introduced at the 3′-terminus of the cDNA amplicon. The DEN-2 virus cDNA amplicon was digested with *Kas*I and *Not*I enzymes and inserted into the *Kas*I and *Not*I sites of a pCBJESS vector to form the 100% DEN-2 E plasmid, pCBD2-14-6. The pCBJESS vector was derived from the pCBamp plasmid which contained the cytomegalovirus

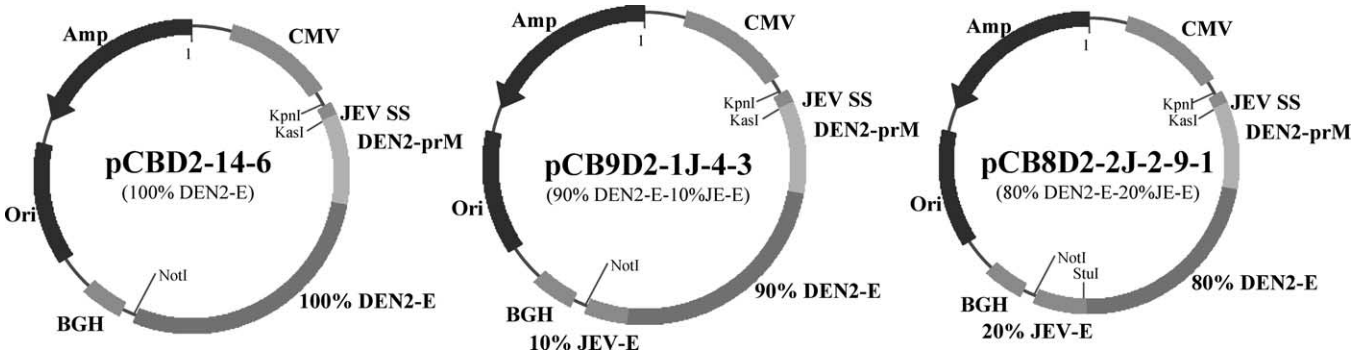

Fig. 2. Schematic representations of plasmid vectors pCBD2-14-6 (100% DEN2-E), PCB9D2-1J-4-3 (90% DEN2-E-10% JE-E), and pCB8D2-2J-2-9-1 (80% DEN2-E-20% JE-E). These plasmids include the human cytomegalovirus (CMV) early gene promoter, JE virus signal sequence, DEN-2 virus prM and E gene region (amino-terminal 100, 90, or 80%), JE virus E gene region (carboxyl-terminal 10 or 20%), and bovine growth hormone poly(A) signal (BGH).

early gene promoter, translational control element, and engineered JE signal sequence element (Davis et al., 2001).

To construct the 90 and 80% DEN-2 E plasmids, the 100% DEN-2 plasmid, pCBD2-14-6, and the JE plasmid, pCBJE1-14, were used as the PCR templates to amplify DEN-2 and JE DNA sequence, respectively (Chang et al., 2000). Primer sets to obtain the DEN-2 and JE gene fragments are listed in Table 4. T7 and SP6 priming sites are found in the pCBamp plasmid, derived from original pcDNA-3 plasmid (Invitrogen, Carlsbad, CA). PCR-amplified DNA fragments for the 90% DEN-2-10% JE E gene were digested with *Bst*XI restriction endonuclease, ligated with T4 DNA ligase, digested with *Kas*I and *Not*I enzymes, and inserted into the *Kas*I and *Not*I sites of the pCBJESS vector to obtain the plasmid pCB9D2-1J-4-3. PCR-amplified DNA fragments for the 80% DEN-2-20% JE E gene were digested with *Bsm*BI, ligated with T4 DNA ligase, digested with *Kas*I and *Not*I enzymes, and inserted into the *Kas*I and *Not*I sites of the pCBJESS vector to obtain the plasmid pCB8D2-2J-2-9-1. Schematic representations of the three plasmid constructs are shown in Fig. 2. The 90% DEN-2-10% JE E and the 80% DEN-2-20% JE E junction regions are shown in Table 4.

Automated DNA sequencing was performed on an ABI Prism 377 Sequencer (Applied Biosystems/Perkin–Elmer, Foster City, CA) according to the manufacturer's recommended procedures. The recombinant plasmids that had correct prM and E sequences were identified.

Transient expression of DEN-2 recombinant antigen in COS-1 cells by electroporation

COS-1 cells were electroporated separately with each DEN-2 plasmid or green fluorescent protein expression plasmid control (pEGFP, Clontech, San Francisco, CA) using the protocol described previously (Chang et al., 2000). Electroporated cells were seeded onto 75-cm^2 culture flasks and incubated at 37°C with 5% CO_2. Six hours following electroporation, the growth medium was replaced with a maintenance medium containing 2% fetal bovine serum. Tissue-culture medium and cells were harvested separately 48 h following electroporation for antigen characterization.

Epitope mapping using DEN-2 E-specific monoclonal antibodies

Forty-eight hours following electroporation, adherent cells were trypsinized, resuspended in phosphate-buffered saline solution (PBS) containing 5% goat serum, spotted on a 12-well spot-slide, and air dried. Cells adhered to the spot-slide were fixed with acetone for 10 min at −20°C and allowed to air dry. E-protein-specific monoclonal antibodies were used to detect protein expression by the indirect IFA, as described previously (Chang et al., 2000).

Characterization of the recombinant DEN-2 virus antigen

Tissue-culture medium was harvested 48 h following electroporation. Antigen-capture (Ag-capture) ELISA was used to detect secreted DEN-2 virus antigen in the culture medium of transiently transformed COS-1 cells. MAb 4G2 and horseradish peroxidase conjugated MAb 6B6C-1 were used to capture the DEN virus antigens and detect captured antigen, respectively (Chang et al., 2000; Hunt et al., 2001).

Recombinant antigen in the medium was concentrated by precipitation with 10% PEG-8000. The precipitant was resuspended in TNE buffer (50 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.5) to 1/100th of the original volume, clarified by centrifugation, and stored at 4°C. Recombinant antigen concentrated by PEG precipitation and resuspended in TNE buffer was extracted with 4% ethanol to remove residual PEG (Hunt et al., 2001). Ethanol-extracted antigens, hydrophobic membrane proteins from the transformed cells, and gradient-purified DEN-2 virions were analyzed under non-denaturing conditions on a NuPAGE, 4-12% Bis–Tris gradient gel in an Excel Plus Electrophoresis Apparatus (Invitrogen Corp.), followed by electroblotting onto nitrocellulose membranes using a Excel Plus Blot Unit (Invitrogen Corp.). DEN-2 virus-specific protein was detected by Western blot using DEN-2 virus-specific MAbs 1A6A-8 (E-specific) and 1A2A-1 (capsid-specific), as well as rabbit antiserum-specific for DEN-2 prM and mouse

serum-specific for a peptide composed of amino acids 1-34 of the DEN-2 M protein, and normal mouse ascitic fluid as a negative control (Murray et al., 1993, Roehrig et al., 1998).

Forty-eight hours following electroporation, transformed cells for each plasmid were trypsinized and aliquots containing 5×10^6 cells were resuspended in PBS. These cell samples were processed for membrane protein extraction using the Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce) following the manufacturer's protocol. Both hydrophobic and hydrophilic proteins were isolated. This procedure was developed for enrichment of integral membrane protein found in the hydrophobic phase. Both hydrophobic and hydrophilic fractions were analyzed by Ag-capture ELISA for DEN-2 recombinant antigen; the hydrophobic fraction was also examined by Western blot as described above.

Mouse vaccination and challenge

Groups of 10 three-week-old female ICR outbred mice were used in the study. Mice were injected im with pCBD2-14-6, pCB9D2-1J-4-3, pCB8D2-2J-2-9-1, or pEGFP on weeks 0 and 3 with a dose of 100 μg in a volume of 100 μl per mouse. The plasmid DNA was purified from XL-1 blue cells with EndoFree Plasmid Giga Kits (Qiagen) and resuspended in PBS, pH 7.5, at a concentration of 1.0 μg/μl. Mice that received 100 μg pEGFP were used as plasmid-vaccinated controls. Mice were bled every 3 weeks following injection, and the DEN-2 virus-specific antibody response was evaluated by indirect ELISA and PRNT.

For evaluation of passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following a single vaccination of pCB8D2-2J-2-9-1 (100 μg/100 μl in PBS) at 3 weeks of age. Pups from unvaccinated females were used as the challenge control. Pups were challenged, ip, at 0 to 2 days after birth with 50,000 PFU/50 μl of NGC virus and observed daily for 3 weeks. Postchallenge serum was collected from mice that survived challenge and was screened by the IgG-ELISA at 1:100 and 1:400.

Serological tests

Pre- and postvaccination serum specimens were tested for antibody binding ability to purified DEN-2 virus by ELISA, for Nt antibody by PRNT, and for antibodies that recognize purified DEN-2 virus proteins by Western blotting. PRNT was performed with Vero cells, as previously described (Chang et al., 2000), using DEN-2 (16681) and JE (Nakayama) viruses. Endpoints were determined at a 90% plaque-reduction level.

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