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A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques

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Abstract

DNA shuffling and screening technologies were used to produce chimeric DNA constructs expressing antigens that shared epitopes from all four dengue serotypes. Three shuffled constructs (sA, sB and sC) were evaluated in the rhesus macaque model. Constructs sA and sC expressed pre-membrane and envelope genes, whereas construct sB expressed only the ectodomain of envelope protein. Five of six, and four of six animals vaccinated with sA and sC, respectively, developed antibodies that neutralized all 4 dengue serotypes in vitro. Four of six animals vaccinated with construct sB developed neutralizing antibodies against 3 serotypes (den-1, -2 and -3). When challenged with live dengue-1 or dengue-2 virus, partial protection against dengue-1 was observed. These results demonstrate the utility of DNA shuffling as an attractive tool to create tetravalent chimeric dengue DNA vaccine constructs, as well as a need to find ways to improve the immune responses elicited by DNA vaccines in general. Published by Elsevier Inc.

Keywords: Dengue vaccines; Chimeric vaccines; DNA vaccines

Introduction

Dengue is one of the most important arboviral diseases of humans. An estimated 100 million dengue infections are caused each year by four antigenically distinct serotypes of dengue viruses (dengue-1 to dengue-4), resulting in widespread dengue fever (DF) and at least 250,000 cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Halstead, 1988). These viruses are transmitted primarily by the mosquito *Aedes aegypti* and are endemic throughout the tropical and subtropical regions of the world. A major expansion of dengue transmission has occurred throughout the tropical Americas accompanied by a large increase in the number of DHF cases. Despite the public health importance of DF and DHF, vector control, which has proven difficult and costly to sustain over time, is the only available control measure (Gubler, 1994). Vaccines are available for two related flaviviruses, yellow fever (YF) virus and Japanese encephalitis (JE) virus (Barrett, 1997a, 1997b). However, decades of effort have not produced a successful dengue virus vaccine. A major obstacle to dengue vaccine development is the epidemiological observation that a dengue virus infection only produces protective immunity against the infecting serotype, and that a secondary infection by a heterologous serotype increases the risk for DHF/DSS. It is postulated that this increased risk for severe dengue disease during secondary infections is due to antibody mediated enhancement (ADE) of infection (Halstead and O'Rourke, 1977), in which virus–antibody complex formed due to pre-existing antibodies from the primary infection gain greater access to target cells via the Fc-receptors. It is therefore imperative that a dengue vaccine produces robust protective immune responses to all 4 dengue serotypes. Conventional methodologies such as live attenuated virus vaccines (Edelman et al., 1994), inactivated dengue viruses (Schlesinger, 1977) and subunit vaccines (Deubel et al., 1988; Eckels et al., 1994) have not yet produced a vaccine. More recently, considerable advances have been made using dengue–yellow fever virus chimeras as vaccine candidates (Guirakhoo et al., 2001, 2002, 2004). However, these methodologies have relied on developing 4

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individual vaccines, one for each serotype, and mixing them to produce the final tetravalent formulation. This approach presents challenges related to commercial scale manufacture and formulation of a human dengue vaccine.

DNA vaccines offer an alternative strategy for developing effective dengue virus vaccines. This approach, in which plasmid DNA expressing an antigen of interest is used as a vaccine, has been shown to induce immune responses in animal models against a number of different viruses, including several flaviviruses (Colombage et al., 1998; Konishi et al., 1998; Phillpotts et al., 1996; Schmaljohn et al., 1997). A West Nile virus (also a flavivirus) DNA vaccine has recently been approved for use in horses (http://www.aphis.usda.gov/lpa/issues/issues_archive/wnv/wnv_vaccine.html), making it the first DNA vaccine to be commercially marketed. We have previously demonstrated the feasibility of DNA vaccines for dengue viruses in a number of studies (Kochel et al., 1997, 2000; Porter et al., 1998; Raviprakash et al., 1995, 2000a, 2000b, 2001, 2003).

In an effort to produce a single DNA vaccine capable of eliciting immune responses to all 4 serotypes, we employed directed molecular evolution by DNA shuffling and screening to generate chimeric vaccine constructs encoding antigens comprising epitopes from all four dengue serotypes. DNA shuffling and screening technologies have previously been used in evolving interferon-α, IL-12, co-stimulatory molecules and viruses (Chang et al., 1999; Lazetic et al., 2002; Leong et al., 2003; Patten et al., 1997; Soong et al., 2000). Using this approach, we generated a number of chimeric DNA vaccines that were shown to contain sequences representative of all 4 dengue serotypes. A number of these chimeric DNA vaccines also produced multivalent neutralizing antibodies in mice and provided protection against intracerebral challenge by dengue-2 virus (Apt et al., 2005). In the present study, we show that 3 selected chimeric DNA vaccines produced by DNA shuffling and screening elicited multivalent dengue virus neutralizing antibodies in vaccinated rhesus macaques. When vaccinated animals were challenged with live virus, partial protection was observed.

Results

Shuffled vaccine candidates

The DNA shuffling and screening methods used to generate and select vaccine candidates that expressed antigens with combined attributes of all four dengue serotypes were recently described (Apt et al., 2005). Based on murine immunogenicity studies (Apt et al., 2005), 3 shuffled clones were selected for evaluation in a nonhuman primate model. The 3 shuffled clones, 6E12-D4, 18H6 and 2G11-D4 (referred to in this study as sA, sB and sC, respectively), all expressed antigens that reacted with all 4 dengue-specific antibodies and elicited antibodies in vaccinated mice that neutralized all 4 dengue serotypes in vitro (Apt et al., 2005). The distribution of amino acid sequences from all 4 dengue serotypes on the hydrophilic surface of each of the 3 shuffled E (envelope) antigens (sA, sB and sC) in a modeled dimer formation (Modis et al., 2003) is shown in Fig. 1.

Immunogenicity in rhesus macaques

Animals were vaccinated by intramuscular injection of 5 mg empty vector DNA , shuffled DNA vaccine sA, sB or sC, a mixture of 1.25 mg each of the 4 wild-type parental DNA (wt-mix) or a mixture of 1.65 mg each of sA, sB and sC DNA (sABC) on days 0, 28 (week 4) and 84 (week 12). Treatment groups and the immunization regimen are shown in Fig. 2. Sera obtained 4 weeks after the first vaccination were diluted 100-fold and dengue-specific IgG antibody was measured by ELISA. All animals (6/6) vaccinated with the wild-type vaccine mixture (wt-mix) had seroconverted after the first dose and elicited antibodies that reacted with all 4 dengue antigens (Fig. 3A). Similarly, a majority of animals vaccinated with sA (4/6), sC (3/6) or sABC (4/6) had seroconverted after first dose producing antibodies reacting to all 4 dengue antigens; remaining animals produced antibodies that reacted with 2 or 3 dengue antigens. Vaccine sB produced the lowest antibody levels after the initial dose, and the antibodies reacted primarily

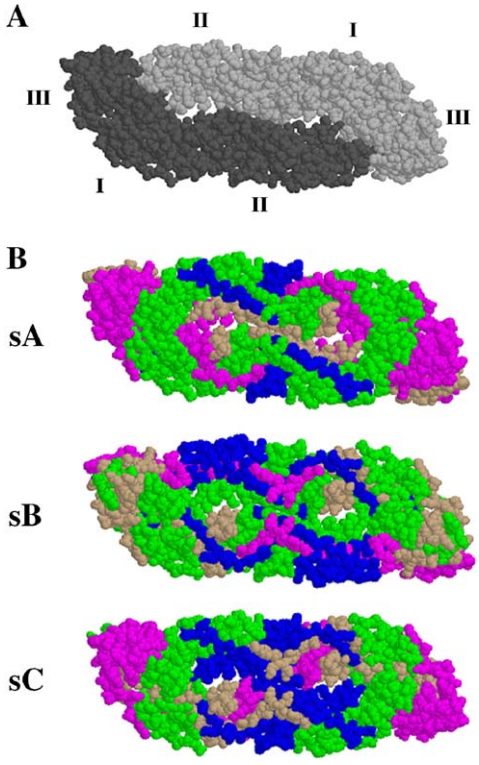

Fig. 1. Space filling models depicting the chimeric nature of the deduced amino acid sequences expressed from shuffled DNA vaccines sA, sB and sC. (A) The hydrophilic face of the soluble dimeric ectodomain from dengue type 2 is shown. The dimer is formed by two anti-parallel E monomers, shown in light gray and dark gray. The 3 domains (I, II and III) of the ectoprotein are outlined. (B) The hydrophilic face of the shuffled E antigens, where amino acid residues from dengue-1 dengue-2, dengue-3 and dengue-4 are shown in magenta, tan, green and blue, respectively. This figure was created with protein explorer (Martz, 2002) using the atomic coordinates 1OAN (Modis et al., 2003) from the protein data bank.

Study Design

A. Vaccine and Challenge Groups

Group	Vaccine (n)	Challenge (n)
1	Vector DNA (5)	Den-1 (2); Den-2 (3)
2	Den1-Den4 Mix (wt-mix) (6)	Den-1 (3); Den-2 (3)
3	sA (6)	Den-1 (3); Den-2 (3)
4	sB (6)	Den-1 (3); Den-2 (3)
5	sC (6)	Den-1 (3); Den-2 (3)
6	sA+sB+sC (6)	Den-1 (3); Den-2 (3)

B. Vaccination and Challenge Schedule

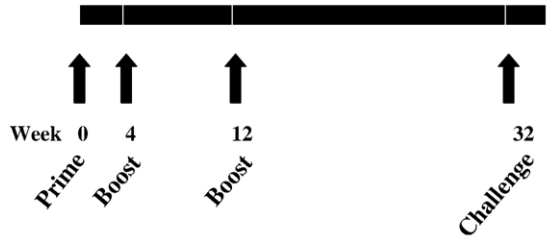

Fig. 2. Study design. (A) Distribution of animals for vaccination and challenge. *n* = number of animals. (B) Vaccination and challenge schedule.

with dengue-1 and dengue-3 antigens. However, after 3 vaccinations, all animals except for the control group produced antibodies reacting with all 4 dengue antigens. Fig. 3B shows mean ELISA optical densities for each group for each dengue antigen, 4 weeks after the last immunization (week 16), measured at 1:1000 serum dilution.

The ability of these antibodies to neutralize virus in vitro was measured by plaque reduction neutralization test (PRNT). All vaccines, except sB, elicited antibodies in a subset of animals (4/6, 5/6, 4/6, 6/6 for wt-mix, sA, sC and sABC, respectively) that neutralized all 4 dengue serotypes in vitro. The geometric mean titers (GMT) for 50% neutralization ($PRNT_{50}$) for all vaccines (day 112 sera) against all 4 dengue serotypes were significantly higher compared to those for the vector control group ($P < 0.05$), with the exception of vaccine sB which elicited antibodies that only neutralized dengue-1, -2 and -3 (Fig. 4). An examination of the neutralization titers also revealed that among the 3 individual shuffled vaccines, vaccine sA performed the best, eliciting mean titers of 27, 23, 58 and 30 against dengue-1, -2, -3 and -4, respectively. However, by day 224 (day of virus challenge), the GMTs for all groups against all dengue serotypes had declined by 50% or more (Fig. 4).

Live virus challenge

Three animals from each group were challenged with live virus by subcutaneous inoculation of 10^5 plaque forming units (PFU) of dengue-1 (strain western pacific 74) or dengue-2 (strain OBS-8041) virus. The vector control group for dengue-1 challenge had 2 animals (1 animal died before the beginning of the protocol due to study unrelated cause). Viremia was assessed by detection of virus in the serum samples of daily bleeds following virus challenge. Table 1 shows a summary of viremia data, as well as neutralizing antibody titers at the time of challenge. Results suggest a partial protection against dengue-1 viremia in vaccinated groups compared to the control group. The mean days of viremia were reduced from 4 (control group) to 1.3, 2.3, 1.3, 2.3 and 0.6 for wt-mix, sA, sB, sC and sABC vaccinated groups, respectively. The reduction in the days of viremia for the sABC group was statistically significant ($P < 0.05$). However, none of the vaccinated groups showed any reduction in dengue-2 viremia. The neutralizing antibody levels at the time of challenge had declined from their observed peak levels at day 112 (Table 1 and Fig. 4), and a clear correlation between neutralizing antibody titers and protection from viremia could not be established.

To determine if the vaccination had primed the animals sufficiently to mount a secondary antibody response to virus challenge, IgM and IgG antibodies were measured by ELISA during the challenge phase (Fig. 5). For dengue-1, the vector control group exhibited a typical primary antibody response in which there was an initial IgM response by day 6 followed by an increasing IgG response by day 10 after challenge. The wt-mix, sB and sC vaccinated animals also showed an IgM response, although their IgGs were boosted at the same time. sA and

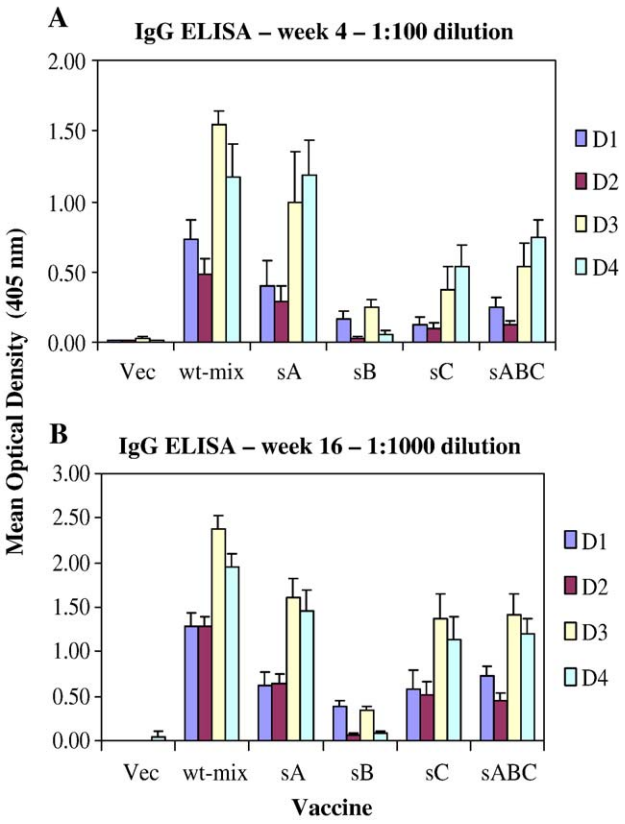

Fig. 3. ELISA reactive antibodies after 1st and 3rd dose of vaccine. Sera were diluted 1:100 (A) or 1:1000 (B) and dengue-specific IgG reacting with each serotype antigen was measured by ELISA (week 4 sera were barely positive at 1:1000 dilution). Mean optical density (405 nm) and standard error for each group against each antigen is shown. All animals were included in the analysis.

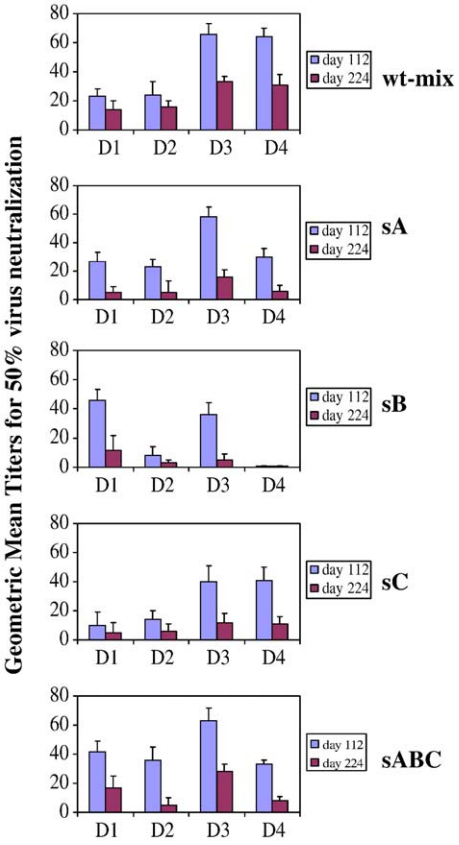

Fig. 4. Virus neutralizing antibodies. Sera from each animal obtained on day 112 (4 weeks after 3rd dose) and on day 224 (day of virus challenge) were used to measure neutralization of dengue-1, -2, -3 and -4 virus by PRNT. A pre-immune serum pool of all the animals was used as the base line. GMT of ($PRNT_{50}$) for each vaccine group for each dengue serotype is shown.

sABC vaccinated animals demonstrated a typical secondary antibody response in which their IgG antibodies increased soon after challenge without significant accumulation of IgM antibodies. Response to dengue-2 challenge was slower; by day 10, IgM antibody was still increasing with little accumulation of IgG antibody in the vector control group. Anti-dengue2 IgM antibody was still rising on day 10 for sB vaccinated animals as well, but unlike vector control group, dengue2-IgG increased simultaneously. Although dengue2-IgG levels at the time of challenge were substantially lower compared to dengue-1 IgG, there was a rapid increase in IgG levels by day 6 without IgM accumulation in wt-mix, sA, sC and sABC vaccinated animals. Measurement of neutralizing antibodies on day 10 after challenge clearly showed a slow primary response for both dengue-1 and dengue-2 challenge in control animals whereas all vaccinated animals exhibited significantly high titers indicating an anamnestic antibody response (Table 2).

Discussion

Development of a dengue vaccine has been complicated by the need to raise protective immune responses against all 4 dengue serotypes. Traditional approaches that depend on using 4 individual dengue virus vaccines mixed to produce a final vaccine have been plagued by constraints of commercial scale manufacture and formulation. In this study, we have taken advantage of the DNA shuffling and screening technology to produce single DNA vaccine candidates that express antigens containing immunogenic epitopes from all 4 dengue serotypes. DNA shuffling and screening is a powerful technology that allows for the selection of clones based on functional attributes in the absence of precise information on the location or structure of the key epitopes. Three shuffled vaccine constructs were chosen for this study based on previous in vitro characterization and in vivo data in a murine model (Apt et al., 2005). All 3 chimeras elicited antibodies in mice that neutralized all 4 dengue serotypes in vitro.

This study demonstrated that single shuffled DNA vaccines sA and sC are capable of producing multiple serotype reactive antibodies in rhesus macaques. The mixture of 4 parental monovalent vaccine constructs and the mixture of the shuffled chimeras also produced antibodies for all serotypes. A tendency for higher antibody response for dengue-3 and dengue-4 compared to dengue-1 and dengue-2 in all vaccinated groups including the wt-mix (Figs. 3 and 4) may indicate inherent differences in the immunogenicity of these E proteins. Shuffled vaccines sA and sC induced neutralizing antibody titers that were comparable to those induced by the vaccine mixtures and caused a reduction in dengue-1 viremia indicating the feasibility of generating multivalent immune responses by single chimeric antigens in macaques. However, the best protection against dengue-1 viremia was demonstrated by the mixture of shuffled vaccines (sABC) and the mixture of parental DNA vaccines (wt-mix). No reduction in viremia was observed in vaccinated animals challenged with dengue-2 virus. It should be noted that the dengue-1 and dengue-2 sequences used for shuffling were derived from published envelope protein sequences of strains AHF 82-80 and D2-04, respectively (Apt et al., 2005). A comparison of E protein sequences of AHF 82-80 and Western Pacific 74 (dengue-1 challenge strain) revealed 7 amino acid substitutions of which 4 were conservative changes. Similarly, 10 amino acid substitutions, of which 4 were conservative changes, were found between D2-04 and OBS-8041 (dengue-2 challenge strain). It is not clear if these substitutions affected the level of protection. It is also possible that shuffling altered the folding of the chimeric envelope proteins.

Virus neutralizing antibodies are of paramount importance in protection against dengue virus (Kliks et al., 1988) and epidemiological data suggest that neutralizing antibodies are sufficient to provide protection against dengue infection. Our measurement of virus neutralizing antibodies showed that shuffled vaccines sA and sC produced virus neutralizing antibodies in macaques to all 4 dengue serotypes, just like the mixture of 4 parental vaccines did. The antibody titers were comparable to those reported for a single dose of recombinant tetravalent live attenuated virus vaccine formulation ($PRNT_{50}$ of 54, 16, 18 and 126 for dengue-1 to dengue-4, respectively) in rhesus macaques (Blaney et al., 2005). These animals were protected against dengue-1, -3 and -4 challenge but not dengue-2. In the study reported

Table 1
Viremia by virus isolation in vero or c6/36 cells

Vaccine	Challenge virus	Animal ID	Day post-challenge								Total days viremia	Mean days viremia	Den-1 PRNT-50 (day 0)	Den-2 PRNT-50 (day 0)
			1	2	3	4	5	6	7	8				
Vector DNA	D1	250										4.0		
		211	+	+	+	+	−	−	−	−	4.0		0	0
		234	+	+	+	+	−	−	−	−	4.0		0	0
	D2	212	+	+	+	+	+	−	−	−	5.0	4.6	0	0
		233	+	+	+	+	+	−	−	−	5.0		0	0
		213	+	+	+	−	+	−	−	−	4.0		0	0
wt Mix	D1	252	−	−	−	−	−	−	−	−	0.0	1.3	12	14
		247	−	−	+	−	−	−	−	−	1.0		0	0
		249	+	−	+	−	+	−	−	−	3.0		33	23
	D2	206	+	+	+	+	+	−	−	−	5.0	5.3	27	16
		225	+	−	+	+	+	+	−	−	5.0		39	25
		235	+	+	+	+	+	+	−	−	6.0		22	32
sA	D1	251	+	+	+	+	−	−	−	−	4.0	2.3	0	10
		240	−	−	+	+	−	−	−	−	2.0		0	16
		236	−	−	+	−	−	−	−	−	1.0		29	51
	D2	210	+	+	+	+	−	−	−	−	4.0	4.6	14	0
		246	+	+	+	+	−	−	−	−	4.0		10	0
		255	+	+	+	+	+	+	−	−	6.0		0	0
sB	D1	227	+	+	+	+	−	−	−	−	4.0	1.3	34	0
		214	−	−	−	−	−	−	−	−	0.0		56	13
		253	−	−	−	−	−	−	−	−	0.0		39	0
	D2	219	+	+	+	+	−	−	−	−	4.0	5.0	0	0
		208	+	+	+	+	+	+	−	−	6.0		48	0
		239	+	+	+	+	+	−	−	−	5.0		0	0
sC	D1	205	−	−	−	−	−	−	−	−	0.0	2.3	33	36
		254	+	+	+	+	−	−	−	−	4.0		0	0
		230	+	−	+	+	−	−	−	−	3.0		0	0
	D2	203	+	+	+	+	+	−	−	−	5.0	5.0	34	10
		217	+	+	+	+	+	+	−	−	6.0		20	19
		228	+	+	+	−	+	−			4.0		0	0
sABC	D1	231	−	−	−	−	−	−	−	−	0.0	0.6	32	0
		229	−	−	+	−	−	−	−	−	1.0		0	0
		258	−	−	+	−	−	−	−	−	1.0		42	0
	D2	223	+	+	+	+	+	+	−	−	6.0	5.0	53	24
		201	+	+	+	+	+	−	−	−	5.0		22	27
		224	+	+	+	+	−	−	−	−	4.0		15	13

Presence of virus in the serum of virus challenged animals was determined for 8 days after challenge as described under Materials and methods. Dengue-1 and dengue-2 viremia were determined by infection of vero and C6/36 cells, respectively. Dengue-1 and dengue-2 challenge virus stocks were produced by propagation in vero and C6/36 cells, respectively, and using these cells for viremia determination increased assay sensitivity (unpublished). '+' and '−' indicate the presence or absence of virus in the serum of each animal for each of the days post-challenge. Also shown for reference are the dengue-1 and dengue-2 virus neutralization titers at the time of challenge (day 0).

here, we have demonstrated a reduction in viremia for dengue-1 but not dengue-2 in animals immunized with shuffled vaccine constructs. The same authors (Blaney et al., 2005) also reported that when dengue-2 neutralizing antibody titer was raised ($PRNT_{50}$ = 174) after a second dose of vaccine, animals were protected from dengue-2 challenge. In another study using recombinant MVA (modified vaccinia, Ankara) expressing dengue-2 truncated E (monovalent vaccine) protein, Men et al. (2000) have reported $PRNT_{50}$ titers of 70 and higher as being protective. Bray et al. (1996) have shown that rhesus monkeys vaccinated with a dengue-2/dengue-4 chimera vaccine (dengue-2 structural genes in an attenuated dengue-4 background) produced dengue-2 virus neutralizing antibody titers of about 640 and were protected from a dengue-2 challenge. Data presented here are consistent with these studies indicating higher neutralizing antibody titers are necessary for protection against dengue-2 challenge. However, in a tetravalent dengue vaccine study using yellow fever virus-dengue chimeras, low antibody titers of 20–80 were also found protective against dengue-2 challenge (Guirakhoo et al., 2004). It is possible that immune responses other than antibody response may be contributing to protection in this case.

It is clear from our study that the animals immunized with DNA vaccines encoding single chimeric dengue antigens provided efficient priming against multiple dengue serotypes. Accumulation of IgM antibody following challenge in some vaccinated animals may be due to presentation of some epitopes by the virus not presented by the chimeric antigen. The chimeric vaccines also provided partial protection against dengue-1

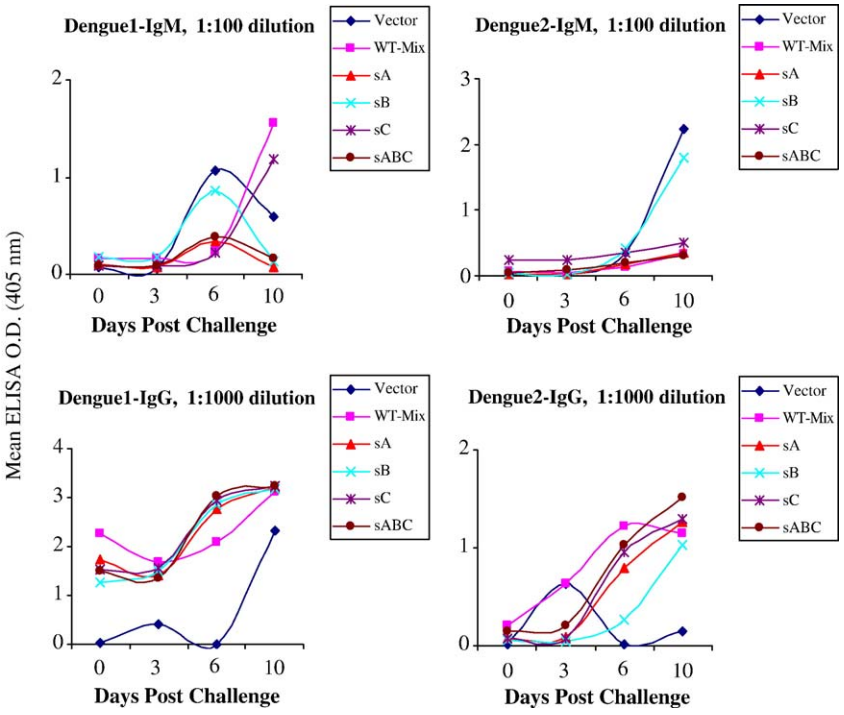

Fig. 5. IgM and IgG antibody responses following virus challenge. Sera obtained on days 0, 3, 6 and 10 (post-challenge) were diluted 1:100 (IgM) or 1:1000 (IgG) and dengue-1 or dengue-2-specific IgM and IgG were measured by ELISA as described in Materials and methods.

virus challenge. This is the first demonstration that the use of DNA shuffling technology can produce vaccine candidates that elicit immune responses against related multiple antigenic proteins in non-human primates. This is important especially to dengue vaccinology where epidemiological data mandate that a vaccine produces protective immune responses to all 4 dengue serotypes. We believe that further investigation to enhance immune responses to these and other shuffled vaccine candidates is warranted.

Table 2
Anamnestic neutralizing antibody response

Vaccine	Challenge virus	Challenge day 0 PRNT-50 (SE)		Challenge day 10 PRNT-50 (SE)	
		Den-1	Den-2	Den-1	Den-2
Vector	Den-1	1 (0)	1 (0)	182 (14)	1 (0)
	Den-2	1 (0)	1 (0)	1 (0)	56 (14)
wt-mix	Den-1	7 (9)	10 (5)	3965 (662)	1178 (616)
	Den-2	28 (5)	23 (4)	4140 (724)	2919 (359)
sA	Den-1	4 (9)	20 (12)	5339 (389)	2021 (969)
	Den-2	5 (3)	1 (0)	2611 (656)	1676 (388)
sB	Den-1	42 (6)	5 (3)	5708 (448)	472 (459)
	Den-2	3 (15)	2 (2)	706 (588)	1863 (335)
sC	Den-1	3 (10)	3 (11)	4369 (884)	1634 (418)
	Den-2	8 (9)	11 (3)	2090 (535)	2002 (587)
sABC	Den-1	11 (12)	1 (0)	4923 (713)	2560 (458)
	Den-2	26 (11)	20 (4)	3474 (767)	3434 (362)

Geometric mean of $PRNT_{50}$ titers are shown for each group at the time of challenge as well as 10 days post-challenge. Undetectable titers (<10) were assigned a value of 1 for purposes of GMT calculation. SE = standard error.

Materials and methods

Animals

Rhesus monkeys (*Macaca mulatta*), of either sex, age 3–8 years, weighing between 2 and 5 kg, were housed at the Naval Medical Research Center/Walter Reed Army Institute of Research animal facility in Silver Spring, MD. The monkeys were prescreened for the presence of dengue-specific antibody by enzyme linked immunosorbant assay (ELISA) and the plaque reduction neutralization test (PRNT). Only those animals that did not show evidence of previous dengue exposure were included in the study. Animals were excluded if the ELISA O.D. exceeded two or more standard deviations at a 1:100 dilution of their sera when tested against a cocktail of dengue antigens (PEG precipitated dengue-1, -2, -3 and -4 virions) compared to the O.D. obtained with a negative control antigen or if the sera (1:10 dilution) exhibited greater than 30% neutralization of input dengue virus (serotypes 1-4) in a standard PRNT.

Vaccines

Construction of the parent and shuffled DNA vaccines has been previously described (Apt et al., 2005). Four parental DNA constructs (D1, D2, D3 and D4) each expressed prM and E genes of dengue-1, -2, -3 and -4, respectively. Two shuffled DNA constructs, sA and sC (clones 6E12-d4 and 2G11-d4, respectively; Apt et al., 2005), expressed prM and E genes in which the N-terminal 90% of E gene was shuffled (contained

portions of sequence representing each of the 4 serotypes). A third shuffled DNA construct sB (clone 18H6; Apt et al., 2005) expressed a shuffled truncated E protein. The DNAs were produced free of endotoxin by Althea Corp., CA.

Immunizations

Groups of 6 animals were immunized by intramuscular injection of 5 mg empty vector DNA, shuffled vaccine A, B or C DNA (sA, sB or sC), a mixture of 1.25 mg each of the 4 wild-type parental DNA (wt-mix) or a mixture of 1.65 mg each of sA, sB and sC DNA (sABC). Immunizations were repeated at weeks 4 and 12. Three milliliters of DNA in PBS was administered at 4 sites (0.65 ml each), one in each of the deltoids and anterior tibialis muscles. Fig. 2 shows the study design indicating different vaccinations and challenges.

Antibody analyses

The monkeys were bled at 4-week intervals, and their sera were tested for dengue serotype-specific antibody by ELISA and/or PRNT. IgG antibody was detected by ELISA as previously described (Ansari et al., 1993) except that polyethylene glycol (PEG) precipitated dengue-1, -2, -3 or -4 virions were used as the antigen, and a peroxidase-labeled anti-human IgG was used as the conjugate. A similar procedure with peroxidase-labeled anti-human IgM was used for IgM determinations. PRNT was performed with vero cells as described (Russell et al., 1967), using two-fold serial dilutions of serum samples. A pool of the pre-immune sera of all the animals was used as the negative control for PRNT. Fifty percent PRNT titers ($PRNT_{50}$) were determined by probit analysis using Minitab software (Minitab Inc, State College, PA).

Virus challenge and viremia

At week 32 (20 weeks after the final dose of vaccine), 3 animals from each vaccine group were challenged with live dengue-1 or dengue-2 virus. Each monkey was bled prior to challenge, then inoculated subcutaneously in the upper left arm with 0.5 ml DMEM containing 10^5 plaque forming units (PFU) of dengue-1 (strain Western Pacific 74) or dengue-2 (strain OBS-8041) virus. The dengue-1 and dengue-2 challenge virus stocks were produced by propagation in vero or C6/36 cells, respectively. These virus doses had earlier been determined to produce uniform viremia of 3–5 days (unpublished). Monkeys were bled daily for 10 days and again on days 21 and 28 after challenge. Sera were used to measure viremia and antibody responses. Viremia was measured by inoculating 25 cm^2 flasks of confluent vero or C6/36 cells in duplicate with 0.2 ml of a 1:10 diluted serum sample. The inoculated cells were incubated for 10 days at 37 °C in a CO_2 incubator. The cells were then scraped off the flasks, washed with PBS and spotted in duplicate onto immunofluorescence slides. Dengue-specific antigen was detected by indirect immunofluorescence using mouse monoclonal antibody 7E11 and FITC-conjugated anti-mouse immunoglobulin. Monoclonal antibody 7E11 is specific to dengue NS-1 protein and is known to bind NS-1 protein of all 4 dengue serotypes (unpublished). Slides were examined under a fluorescent microscope with appropriate positive and negative controls.

Statistical analyses

Log transformed PRNT-50 titers for each of the 4 dengue serotypes were analyzed by one-way ANOVA for equality among different vaccine treatments. Animals that had titers of zero were assigned a value of 1 for log transformation purpose. Following one-way ANOVA, comparison of multiple means was performed using Fisher's LSD test. Because all the animals in the vector control group had titers of zero indicating no variability, thus nullifying the assumption of equal variances among groups, Fisher's LSD test was repeated excluding the vector control group from analysis. However, both analyses (with and without control group) produced identical results. Similarly, for virus challenge data, mean days of viremia for dengue-1 and dengue-2 were analyzed separately by one-way ANOVA and Fisher's LSD test.

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References

Ansari, M.Z., Shope, R.E., Malik, S., 1993. Evaluation of vero cell lysate antigen for the ELISA of flaviviruses. J. Clin. Lab. Anal. 7 (4), 230–237.

Apt, D., Raviprakash, K., Brinkman, A., Semyonov, A., Yang, S., Skinner, C., Diehl, L., Lyons, R., Porter, K., Punnonen, J., 2005. Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. Vaccine, 335–344.

Barrett, A.D., 1997a. Japanese encephalitis and dengue vaccines. Biologicals 25 (1), 27–34.

Barrett, A.D., 1997b. Yellow fever vaccines. Biologicals 25 (1), 17–25.

Blaney Jr., J.E., Matro, J.M., Murphy, B.R., Whitehead, S.S., 2005. Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys. J. Virol. 79 (9), 5516–5528.

Bray, M., Men, R., Lai, C.J., 1996. Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. J. Virol. 70 (6), 4162–4166.

Chang, C.C., Chen, T.T., Cox, B.W., Dawes, G.N., Stemmer, W.P., Punnonen, J., Patten, P.A., 1999. Evolution of a cytokine using DNA family shuffling. Nat. Biotechnol. 17 (8), 793–797.

Colombage, G., Hall, R., Pavy, M., Lobigs, M., 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology 250 (1), 151–163.

Deubel, V., Kinney, R.M., Esposito, J.J., Cropp, C.B., Vorndam, A.V., Monath, T.P., Trent, D.W., 1988. Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. J. Gen. Virol. 69 (Pt. 8), 1921–1929.

Eckels, K.H., Dubois, D.R., Summers, P.L., Schlesinger, J.J., Shelly, M., Cohen, S., Zhang, Y.M., Lai, C.J., Kurane, I., Rothman, A., et al., 1994. Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. Am. J. Trop. Med. Hyg. 50 (4), 472–478.

Edelman, R., Tacket, C.O., Wasserman, S.S., Vaughn, D.W., Eckels, K.H., Dubois, D.R., Summers, P.L., Hoke, C.H., 1994. A live attenuated dengue-1 vaccine candidate (45AZ5) passaged in primary dog kidney cell culture is attenuated and immunogenic for humans. J. Infect. Dis. 170 (6), 1448–1455.

Gubler, D.J., 1994. Perspectives on the prevention and control of dengue hemorrhagic fever. Gaoxiong Yixue Kexue Zazhi 10, S15–S18 (Suppl.).

Guirakhoo, F., Arroyo, J., Pugachev, K.V., Miller, C., Zhang, Z.X., Weltzin, R., Georgakopoulos, K., Catalan, J., Ocran, S., Soike, K., Ratterree, M., Monath, T.P., 2001. Construction, safety, and immunogenicity in non-human primates of a chimeric yellow fever-dengue virus tetravalent vaccine. J. Virol. 75 (16), 7290–7304.

Guirakhoo, F., Pugachev, K., Arroyo, J., Miller, C., Zhang, Z.X., Weltzin, R., Georgakopoulos, K., Catalan, J., Ocran, S., Draper, K., Monath, T.P., 2002. Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 298 (1), 146–159.

Guirakhoo, F., Pugachev, K., Zhang, Z., Myers, G., Levenbook, I., Draper, K., Lang, J., Ocran, S., Mitchell, F., Parsons, M., Brown, N., Brandler, S., Fournier, C., Barrere, B., Rizvi, F., Travassos, A., Nichols, R., Trent, D., Monath, T., 2004. Safety and efficacy of chimeric yellow fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J. Virol. 78 (9), 4761–4775.

Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239 (4839), 476–481.

Halstead, S.B., O'Rourke, E.J., 1977. Antibody-enhanced dengue virus infection in primate leukocytes. Nature 265 (5596), 739–741.

Kliks, S.C., Nimmanitya, S., Nisalak, A., Burke, D.S., 1988. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am. J. Trop. Med. Hyg. 38 (2), 411–419.

Kochel, T., Wu, S.J., Raviprakash, K., Hobart, P., Hoffman, S., Porter, K., Hayes, C., 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine 15 (5), 547–552.

Kochel, T.J., Raviprakash, K., Hayes, C.G., Watts, D.M., Russell, K.L., Gozalo, A.S., Phillips, I.A., Ewing, D.F., Murphy, G.S., Porter, K.R., 2000. A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in Aotus monkeys. Vaccine 18 (27), 3166–3173.

Konishi, E., Yamaoka, M., Khin Sane, W., Kurane, I., Mason, P.W., 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. J. Virol. 72 (6), 4925–4930.

Lazetic, S., Leong, S.R., Chang, J.C., Ong, R., Dawes, G., Punnonen, J., 2002. Chimeric co-stimulatory molecules that selectively act through CD28 or CTLA-4 on human T cells. J. Biol. Chem. 277 (41), 38660–38668.

Leong, S.R., Chang, J.C., Ong, R., Dawes, G., Stemmer, W.P., Punnonen, J., 2003. Optimized expression and specific activity of IL-12 by directed molecular evolution. Proc. Natl. Acad. Sci. U.S.A. 100 (3), 1163–1168.

Martz, E., 2002. Protein explorer: easy yet powerful macromolecular visualization. Trends Biochem. Sci. 27 (2), 107–109.

Men, R., Wyatt, L., Tokimatsu, I., Arakaki, S., Shameem, G., Elkins, R., Chanock, R., Moss, B., Lai, C.J., 2000. Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge. Vaccine 18 (27), 3113–3122.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 100 (12), 6986–6991.

Patten, P.A., Howard, R.J., Stemmer, W.P., 1997. Applications of DNA shuffling to pharmaceuticals and vaccines. Curr. Opin. Biotechnol. 8 (6), 724–733.

Phillpotts, R.J., Venugopal, K., Brooks, T., 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. 141 (3–4), 743–749.

Porter, K.R., Kochel, T.J., Wu, S.J., Raviprakash, K., Phillips, I., Hayes, C.G., 1998. Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. Arch. Virol. 143 (5), 997–1003.

Raviprakash, K., Liu, K., Matteucci, M., Wagner, R., Riffenburgh, R., Carl, M., 1995. Inhibition of dengue virus by novel, modified antisense oligonucleotides. J. Virol. 69 (1), 69–74.

Raviprakash, K., Kochel, T.J., Ewing, D., Simmons, M., Phillips, I., Hayes, C.G., Porter, K.R., 2000a. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine 18 (22), 2426–2434.

Raviprakash, K., Porter, K.R., Kochel, T.J., Ewing, D., Simmons, M., Phillips, I., Murphy, G.S., Weiss, W.R., Hayes, C.G., 2000b. Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J. Gen. Virol. 81 (Pt. 7), 1659–1667.

Raviprakash, K., Marques, E., Ewing, D., Lu, Y., Phillips, I., Porter, K.R., Kochel, T.J., August, T.J., Hayes, C.G., Murphy, G.S., 2001. Synergistic neutralizing antibody response to a dengue virus type 2 DNA vaccine by incorporation of lysosome-associated membrane protein sequences and use of plasmid expressing GM-CSF. Virology 290 (1), 74–82.

Raviprakash, K., Ewing, D., Simmons, M., Porter, K.R., Jones, T.R., Hayes, C.G., Stout, R., Murphy, G.S., 2003. Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys. Virology 315 (2), 345–352.

Russell, P.K., Nisalak, A., Sukhavachana, P., Vivona, S., 1967. A plaque reduction test for dengue virus neutralizing antibodies. J. Immunol. 99 (2), 285–290.

Schlesinger, R.W., 1977. Dengue viruses. Virol. Monogr. (16), 1–132.

Schmaljohn, C., Vanderzanden, L., Bray, M., Custer, D., Meyer, B., Li, D., Rossi, C., Fuller, D., Fuller, J., Haynes, J., Huggins, J., 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. 71 (12), 9563–9569.

Soong, N.W., Nomura, L., Pekrun, K., Reed, M., Sheppard, L., Dawes, G., Stemmer, W.P., 2000. Molecular breeding of viruses. Nat. Genet. 25 (4), 436–439.