CHIMERIC DENGUE TYPE 2/TYPE 1 VIRUSES INDUCE IMMUNE RESPONSES IN CYNOMOLGUS MONKEYS

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Abstract. Chimeric dengue type 2/type 1 (DEN2/1) viruses, which contain the structural genes of the dengue-1 (16007) parental virus and the nonstructural genes of the DEN2-PDK53 virus, have been constructed. These DEN2/1 viruses induce high levels of DEN1 virus-specific neutralizing antibodies in mice. In this study, the DEN2/1 viruses induced DEN1 virus-specific neutralizing antibodies without the development of viremia in cynomolgus monkeys. Dengue virus-specific IgM antibodies were detected in the sera of the immunized animals as early as 3 days post-immunization. After challenge with the DEN1-16007 wild-type virus, only a low level of viremia was detected in chimeric DEN2/1 virus-immunized monkeys. A second challenge, with DEN2-16681 virus, was given while the levels of DEN2-specific neutralizing antibodies were very low: infectious Dengue 2 virus could not be detected in sera of the monkeys. A correlation between the level of neutralizing antibody and the incidence of viremia could not be found. In addition, there was no significant increase in the levels of interferon gamma and soluble interleukin 2 receptor in the sera of the challenged monkeys, which suggests a reduction in immunopathogenesis caused by T-cell activation. Our findings suggest that DEN2/1 viruses may used as a live-attenuated candidate vaccine because of their safety, broad immunogenicity, and lower immunopathogenicity.

INTRODUCTION

Dengue (DEN) viruses are members of the *Flaviviridae* family. There are 4 serotypes (DEN1-4). Infection with these viruses has caused high fatality rates in children in tropical and sub-tropical areas (Gubler, 1998). Dengue virus infection causes two forms of illness, dengue fever (DF) and dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). DF is a self-limiting disease; on the other hand, DHF/DSS is a life-threatening condition: the leakage of plasma into the interstitial space may result in hypovolemic shock (Halstead, 1988; Hayes and Gubler, 1992). It has been proposed that serotype cross-reactive immune responses contribute to the immunopathogenesis of DHF/DSS following secondary infection with a heterologous serotype of DEN virus (Kurane and Ennis, 1994). An ideal DEN vaccine would induce protective immune responses to all 4 serotypes of DEN virus, while avoiding immunopathogenesis in vaccinees.

The Center for Vaccine Development, Mahidol University, has developed live attenuated candidate vaccines for the four DEN serotypes. The DEN2-PDK53 vaccine virus, which has been evaluated in human trials in Thailand and in the USA, is strongly immunogenic and safe in humans (Bhamarapravati *et al*, 1987; Dharakul *et al*, 1994; Vaughn *et al*, 1996). On the other hand, the candidate

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DEN1-PDK13 vaccine virus, while requiring the highest infectious dose for the immunization of humans, produces lower seroconversion rates (Yoksan *et al*, 1986). A booster dose of the DEN1-PDK13 virus was needed to achieve 100% seroconversion.

Infectious cDNA clones of the DEN2-PDK53 virus have been constructed and evaluated (Kinney *et al*, 1997; Butrapet *et al*, 2000). Attenuation markers of the DEN2-PDK53 vaccine virus, including limited replication in C6/36 cells, small plaque size, temperature sensitivity, and attenuation for newborn mice, are determined by mutations located in nonstructural regions, including the 5′-noncoding region-57 (5′-NCR-57) C-to-T (16681-to-PDK53), NS1-53 Gly-to-Asp, and NS3-250 Glu-to-Val, of the DEN viral genome (Butrapet *et al*, 2000). Because the virus-attenuating mutations reside in the nonstructural regions of the DEN2-PDK53 genome, the DEN2-PDK53 virus may serve as a unique vector for serve as a unique vector for the construction of chimeric DEN viruses that express structural genes of DEN-1, DEN-3, DEN-4, or other flaviviruses.

Chimeric DEN2/1 viruses that might be improved alternatives to the DEN1-PDK13 vaccine virus have been constructed (Huang *et al*, 2000). Chimeric DEN2/1 viruses, in which the C-prM-E genes of the DEN2-PDK53 virus had been replaced by those of the DEN1-16007 parental virus, have been developed and tested for their *in vitro* replication markers, neurovirulence in newborn mice, and immunogenicity in weanling mice. Mice immunized with these DEN2/1 chimeras developed significantly greater primary and boosted PRNT titers than mice immunized with the DEN1-PDK13 vaccine virus (Huang *et al*, 2000).

Small animals have been widely used for evaluating the safety and immunogenicity of DEN vaccine candidates. However, the results of these studies are difficult to interpret because DEN virus usually is not pathogenic in the animals that are used (Raut *et al*, 1996). Although an appropriate animal model has not been developed for testing DEN vaccines, monkeys have been used to study the biology of DEN and other flaviviruses (Rosen, 1958; Rodhain, 1991). The DEN vaccine candidates that have been developed at Mahidol University have their safety and neurovirulence evaluated in monkeys (Angsubhakorn, *et al*, 1986, 1987a, 1987b, 1988, 1994). In this study, we evaluated the immune responses and viremia of adult cynomolgus monkeys that had been immunized with chimeric DEN2/1 viruses.

Cytotoxic T-lymphocytes (CTLs) play a role during DEN virus infection. As the Flavivirus NS3 protein is a major target of the DEN virus-specific and serotype-cross-reactive T-cell responses, the possibility of stimulating immunopathology via a T-cell response cannot be ruled out. CTL studies in DEN virus infected mice and humans suggest that CTL responses can be influenced by the viral serotype (Rothman *et al*, 1996; Spaulding *et al*, 1999). Epidemiological studies have suggested that the order of acquisition of DEN virus infections is important. When DEN2 virus is the cause of a secondary infection, the risk of DHF or DSS appears to be greater (Sangkawibha *et al*, 1984; Thein *et al*, 1997). Because the chimeric DEN2/1 viruses contain the non-structural genes of the DEN2-PDK53 virus, it is important to study the effect of the DEN2 virus-specific nonstructural genes on the T-cell responses of chimera-immunized vaccinees who are subjected to a secondary challenge with DEN viruses. Cytokine levels during T-cell responses to secondary infection with DEN2 may also be significant: they too were assessed in this study.

MATERIALS AND METHODS

Animals

Adult crab-eating cynomolgus monkeys (*Macaca fascicularis*) were donated by the Department of Veterinary Medicine, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok. The monkeys were transferred from AFRIMS and housed in the animal facility of the Center for Vaccine Development, Mahidol University at Salaya, for one month. The experiments were conducted according to

the guidelines for animal ethics published by National Research Council of Thailand and the principles in the Guide for the Care and Use of Laboratory Animals (Svendsen and Hau, 1994). Eight monkeys with no detectable neutralizing antibody against any of the 4 serotypes of DEN virus and Japanese encephalitis (JE) virus were selected for the test. Two groups of three monkeys were immunized; two monkeys served as the non-immunized control group. The subjects were fasted for 15 hours prior to the experiment and given ketamine hydrochloride (10 mg/kg) before bleeding. After immunization, inoculation, and bleeding, the animals were closely observed for at least 30 minutes for signs of discomfort.

Viruses

All virus seeds were grown in LLC-MK_2 cell culture. Two types of chimeric DEN2/1 virus were tested: DEN2/1-EP and DEN2/1-VP, whose construction has been described by Huang *et al* (2000). Briefly, the structural genes (C/prM/E) of parental DEN1-16007 virus were engineered into both variants of the DEN2-PDK53 virus-specific cDNA. Chimeric DEN2/1-EP virus has the DEN2-PDK53-E genetic background and DEN2/1-VP virus contains the PDK53-V backbone. The phenotypic and attenuation markers of these chimeric DEN2/1 viruses have been reported previously (Butrapet *et al*, 2000; Huang *et al*, 2000). The lots of virus seeds were validated by reverse transcriptase PCR and nucleotide sequencing of the entire viral genome. The E (Glu) and V (Val) genetic backgrounds refer to the two major genetic variants (NS3-250-Glu and NS3-250-Val) that are present in the DEN2-PDK53 vaccine (Kinney *et al*, 1997). Parental viruses (DEN1-16007, DEN2-16681, DEN3-16562 and DEN4-1036) and JE-Beijing1 were used in the serological testing; DEN1-16007 and DEN2-16681 were the challenge viruses.

Immunization and challenge

On day 1, the monkeys were immunized subcutaneously in the upper shoulder area with a total of 10^6 PFU of chimeric DEN2/1virus diluted to final volume of 1.0 ml with BA-1 diluent: three monkeys (AF152, AF154 and AF181) were immunized with DEN2/1-EP virus; monkeys AF143, AF165, and AF170 were immunized with DEN2/1-VP virus. The control monkeys (AF179 and AF188) were injected with 1.0 ml of BA-1 diluent. The monkeys were monitored closely during the 30 minutes after immunization, and then daily, for signs of illness. A booster dose of 10^6 PFU of the DEN2/1 viruses was given on day 93 (Table 1).

Two separate virus challenges were conducted (Table 1). First, all the monkeys were challenged with 10^6 PFU of parental DEN1-16007 virus on day 107; second, monkeys AF 179 (non-immunized), AF 165 and AF170 (DEN2/1-VP virus-immunized), and AF 152 and AF154 (DEN2/1-EP virus-immunized) were challenged with 10^6 PFU of DEN2-16681 virus on day 253. Monkeys AF188 (control), AF143 (DEN2/1-VP), and AF181 (DEN2/1-EP) were inoculated on day 253 with BA-1 diluent.

Viremia and antibody analyses

The monkeys were bled on days 3, 5, 7, 9, 11 and 14 and then at monthly intervals; they were also bled on days 3, 5, 7, 9, 11 and 14 after the day of virus challenge. Viremia levels in these sera were tested by RT-PCR, direct plaque titration of 0.1 ml of serum in Vero cell monolayers in duplicate, and plaque titration of clarified culture medium from LLC-MK_2 cells inoculated in duplicate with 0.1 ml of serum and incubated for 7 days at 37ºC. The plaque titration method was that outlined by Butrapet *et al* (2000).

To measure viremia by RT-PCR, 50 µl of each serum sample was inoculated into confluent LLC-MK_2 cells grown in 3.5 cm (diameter) tissue culture dishes. The inoculated cells were incubated at 37ºC for 7 days in a CO_2 incubator, after which the supernatant was collected and clarified by centrifugation. Template viral RNA was extracted from 200 µl of clarified supernatant as well as directly from monkey sera. RT-PCR was performed for each RNA extract. Positive RT-PCR results for the clarified cell culture medium or monkey serum was

considered as positive for viremia. RT-PCR was performed with DEN1-16007 or DEN2-16681 virus-specific primers:

D2-17	5′-GGA CCG ACA AAG ACA GAT TCT TTG A-3′
CD1-349	5′-CCC GTA ACA CTT TAA TCG CTC CAT TC-3′
D1-1395	5′-TGA GAC TAC AGA ACA TGG AAC AAC T-3′
CD1-1923	5′-TTC ATA TTT AAC CTG CAC CAG AAC T-3′
D2-1750	5′-ATC CAA ATG TCA TCA GGA AAC T-3′
CD2-2108	5′-AGC TTC AGT TGT CCC GGC T-3′

DEN virus-specific antibodies were assayed by indirect enzyme-linked immunosorbent assay (ELISA) and plaque-reduction neutralizing testing (PRNT). ELISA was performed by using dengue fever virus ELISA IgM kits (MRL Diagnostics, CA, USA). PRNT of a series of two-fold serial dilutions of sera was performed in LLC-MK_2 monolayers as described by Huang *et al* (2000). Monkey sera collected before immunization served as negative controls for the PRNT. Antibody titers were defined as the antibody dilution that neutralized ≥ 50% virus used in the test ($PRNT_{50}$); titers were considered to be negative if a 1:10 dilution of the serum resulted in less than a 50% reduction of the input virus.

Evaluation of T-cell function

T-cell activation was evaluated by measuring levels of IL-2 sRα and IFN-γ in monkey sera after the second challenge with DEN2-16681 virus using the Human IL-2 sRα and IFN-γ Immunoassay kit (Quantikine, R&D Systems, MN, USA).

RESULTS

Cynomolgus monkeys were transferred from AFRIMS and housed in the CVD animal facility for one month before they joined the experiment. Monkeys were scheduled for immunization and virus challenge as shown in Table 1. The animals showed no signs of any illness after immunization or virus challenge. Their body weights were stable and their body temperatures showed no significant changes throughout the course of the study (data not show).

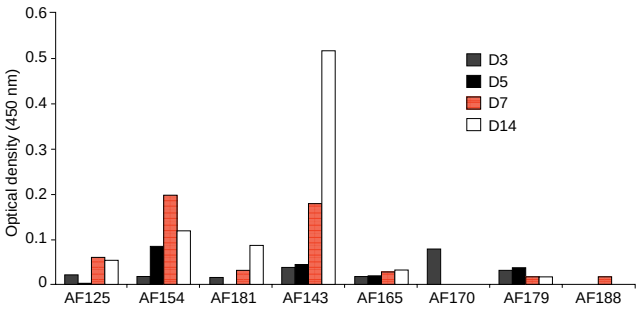

Fig 1–Level of anti-DEN specific IgM antibody in cynomolgus monkey sera detected by indirect ELISA.

Viremias and immune responses following immunization with chimeric DEN-2/1 viruses

No viremia was detected by direct virus plaque assays of the sera in Vero cell monolayers or by isolation attempts in LLC-MK_2 cell culture. Neither DEN2/1-EP nor DEN2/1-VP virus caused a detectable viremia after immunization. DEN viral-specific IgM was detected in 5 of 6 immunized monkeys as early as 3 days after primary immunization by ELISA (Fig 1). At 30 days after primary immunization, the sera of all six DEN2/1 virus-immunized monkeys showed DEN1 virus-specific neutralizing antibodies ($PRNT_{50}$) of reciprocal titer 20-320 (Table 2). Except for monkeys AF181 (DEN2/1-EP) and AF165 (DEN2/1-VP), which had reciprocal titers of 320 and 20 respectively as both days 30 and 90 after primary immunization, neutralizing antibody titers in the monkeys declined at titers of 80 and 10, respectively at day 110 after primary immunization. However, after a booster dose of DEN2/1-EP or DEN2/1-VP virus on day 93 after the primary immunization, followed by a challenge with 10^6 PFU of wild-type DEN1-16007 virus

Table 1
Immunization and virus challenge schedule.

Day	0	1	93	107	253
Action	Screen sera	DEN2/1 virus immunization	DEN2/1 booster dose	DEN1-16007 virus challenge	DEN2-16681 virus challenge
AF152[a]		DEN2/1-EP	DEN2/1-EP	DEN1	DEN2
AF154		DEN2/1-EP	DEN2/1-EP	DEN1	DEN2
AF181		DEN2/1-EP	DEN2/1-EP	DEN1	BA-1
AF143		DEN2/1-VP	DEN2/1-VP	DEN1	BA-1
AF165		DEN2/1-VP	DEN2/1-VP	DEN1	DEN2
AF170		DEN2/1-VP	DEN2/1-VP	DEN1	DEN2
AF179		BA-1	BA-1	DEN1	DEN2
AF188		BA-1	BA-1	DEN1	BA-1

[a]Monkey identification code.

on day 107 after primary immunization (14 days after the booster immunization), the levels of neutralizing antibodies against DEN1 virus in all 6 chimera-immunized monkeys dramatically increased to reciprocal titers of 1,280-5,120 (measured at day 110 following the primary immunization and 3 days after challenge with DEN1-16007 virus; see post-booster titers in Table 2. No significant DEN1 or DEN2 virus-specific neutralizing antibodies were detected in the sera of the control monkeys AF179 and AF188. These data demonstrated that immunization of monkeys with the chimeric DEN2/1 virus was able to induce specific neutralizing antibody against DEN1 virus.

Viremias following challenge with DEN1-16007 viruses

The control monkeys developed low-level viremia, which was detectable on day 9 (monkey AF188) or day 14 (AF179) after challenge with DEN1 virus (Table 3). Viremia was not detected by RT-PCR in 2 monkeys, AF154 (DEN2/1-EP) and AF170 (DEN2/1-VP) (Table 3). However, viremia was detected by RT-PCR at least once in the other 4 immunized animals. Monkeys AF152 and AF181 both had RT-PCR-detectable viremia on a single day (day 9) after challenge with DEN1-16007 virus (Table 3). This one day of viremia occurred in monkey AF181 despite the animal's high reciprocal $PRNT_{50}$ titer of 320 on day 90 (Table 2). Monkeys AF143 and AF165 had RT-PCR-detectable viremias of longer duration (days 11 and 14 and 9, 11 and 14 respectively). No correlation was observed between neutralizing antibody titer and the absence of viremia. The control monkeys showed a low incidence of viremia following DEN1 virus challenge, and there was a general lack of correlation between neutralizing antibody titer and incidence of viremia. Therefore, in terms of reduced viremia following challenge with DEN1 virus, we were unable to identify a protective effect of immunization with the chimeric DEN2/1 viruses.

Immune response and viremia after challenging with a second virus, DEN2 virus

DEN2-16681 virus-specific RNA was detected by RT-PCR in the serum of the DEN2 virus-challenged monkeys on day 5 (monkeys AF152, AF154, and AF165) and on days 3 and 5 (AF170 and AF179) after inoculation with the DEN2 virus (Table 3). However, DEN2 virus-specific RNA was not detected in clarified culture media harvested from serum-inoculated LLC-MK_2 cells (not shown), which indicated that there was not detectable infectious DEN2 virus in the serum of any of the chal-

lenged monkeys. Immediately prior to challenge with DEN2 virus, all of the monkey sera showed significant reciprocal $PRNT_{50}$ titers (80-640) against DEN1-16007 virus, undetectable to significant levels (0-160) of neutralizing antibodies against DEN2-16681 virus, and undetectable to low titers against DEN3-16562 (10-40), DEN4-1036 (0-10), and JE (0-10) viruses (Table 4). The sera of five monkeys (AF152, AF154, AF165, AF170, and AF179) showed undetectable to low reciprocal $PRNT_{50}$ anti-DEN2 titers of 0-20 before DEN-2 virus challenge. $PRNT_{50}$ titers against DEN1, DEN2, and DEN3 viruses were generally higher in the monkeys (AF152, AF154, AF165, AF170, and AF179) that were challenged with DEN2 virus than in the monkeys (AF181, AF143, AF188) that received BA-1 diluent. Except for monkey AF165, which showed a decrease in reciprocal anti-DEN1 titer from 1,280 to 80, the anti-DEN1 and anti-DEN2 titers of all of the monkeys were stable between 30 and 60 days following challenge with DEN2 virus.

Only monkey AF154 (immunized with DEN2/1-EP virus) showed an increasing level (up to 10-fold) of serum IFN-γ 3-7 days after DEN2 virus challenge, relative to the IFN-γ level just prior to DEN2 virus challenge (Table 5). The level of IL-2 sRα in the sera of all of the monkeys failed to increase or increased less than two-fold, relative to levels immediately prior to challenge with the DEN2 virus (Table 5). In DHF patients, level of IL-2 sRα may increase by more than 3-fold; IFN-γ may be increased 4- to 14-fold (Kurane *et al,* 1991, 1995).

DISCUSSION

We have demonstrated that chimeric DEN2/1-EP and DEN2/1-VP viruses elicit DEN1 virus-specific neutralizing antibody responses in cynomolgus monkeys in the

Table 2
Neutralizing antibody responses in cynomolgus monkeys after immunization with chimeric DEN2/1 viruses.

		Reciprocal neutralizing antibody titer (50%)[a]									
		vs DEN1-16007 virus					*vs* DEN2-16681 virus				
Immunization[b]	Animal	Day 0	Day 30	Day 60	Day 90	Post booster[c]	Day 0	Day 30	Day 60	Day 90	Post booster
DEN2/1-EP	AF 152	-	80	20	-	2,560	-	-	-	-	40
	AF 154	-	20	-	-	5,120	-	-	-	-	-
	AF 181	-	320	320	320	2,560	-	-	-	-	-
DEN2/1-VP	AF 143	-	160	80	20	1,280	-	-	-	-	-
	AF 165	-	20	20	20	2,560	-	-	-	-	-
	AF 170	-	40	40	10	2,560	-	-	-	-	10
BA-1	AF 179	-	-	-	-	-	-	-	-	-	-
	AF 188	-	-	-	-	-	-	-	-	-	-

[a]Dilution of serum which resulted in 50% reduction of the input DEN1-16007 PFU .
[b]Monkeys were immunized with 10^6 PFU of chimeric DEN2/1-EP or -VP virus. Days are time after primary immunization.
[c]Post booster refers to day 110 after primary immunization and 17 days after boosting with chimeric DEN 2/1 viruses (3 days after challenge with DEN1-16007 virus).
- = lowest dilution of serum (1:10) resulted in less than 50% reduction of the input virus PFU.

absence of viremia detectable by both RT-PCR and culture. Neutralizing antibody titers against DEN1-16007 virus increased dramatically in all six monkeys following a booster immunization and challenge with DEN1-16007 virus. Following challenge with DEN1-16007 virus, the non-immunized control monkeys failed to develop significant patterns of viremia and, therefore, we were unable to determine the protective efficacy of immunization with the chimeric viruses. Challenge of the DEN2/1 virus-immunized monkeys with DEN2-16681 virus caused either no change to or a slight increase in levels of IFN-γ and IL2-sRα in the sera; no sign of any serious illness was observed.

DEN2/1-EP and DEN2/1-VP viruses, which express the structural genes of DEN1-16007 virus within the genetic background of the two DEN2-PDK53 variants, appeared to be potential DEN1 vaccine candidates. The presence of neutralizing antibodies in DEN-2/1-immunized monkeys (this study) and in mice (Huang *et al*, 2000) indicated that these chimeric viruses are immunogenic. Our results also demonstrated the potential safety of the chimeric DEN2/1-EP and -VP viruses, which failed to produce detectable viremias in the monkeys after primary immunization. In this study, the protective effect of immunization with chimeric DEN2/1 viruses, as measured by the incidence of RT-PCR-detectable viremia following challenge with DEN1-16007 virus was indeterminate or ambiguous, largely because a minimal incidence of DEN1 viremia was detected in the non-immunized control monkeys. However, following challenge with DEN1-16007 virus, monkeys immunized with DEN2/1-EP virus produced RT-PCR-detectable viremias that appeared to be of somewhat shorter duration than those of the monkeys immunized with the DEN2/1-VP virus. This may imply that the DEN2/1-EP virus might be a more effective DEN1 vaccine candidate than the DEN2/1-VP

Table 3
Viremia in cynomolgus monkeys after challenge with DEN1-16007 virus and DEN2-16681 virus.

Immunization	Monkeys	Time post challenge with DEN1-16007 virus[a]						Time post challenge with DEN2-16681[b]					
		Day 3	Day 5	Day 7	Day 9	Day 11	Day 14	Pre-challenge[c]	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
DEN2/1-EP	AF 152	-	-	-	+	-	-	-	-	+	-	-	-	-
	AF 154	-	-	-	-	-	-	-	-	+	-	-	-	-
	AF 181	-	-	-	+	-	-	-	-	-	-	-	-	-
DEN2/1-VP	AF 143	-	-	-	-	+	+	-	-	-	-	-	-	-
	AF 165	-	-	-	+	+	+	-	-	+	-	-	-	-
	AF 170	-	-	-	-	-	-	-	+	+	-	-	-	-
BA-1	AF 179	-	-	-	-	-	+	-	+	+	-	-	-	-
	AF 188	-	-	-	+	-	-	-	-	-	-	-	-	-

[a]Days are time after the first challenge with DEN1-16007 virus.
[b]Days are time after the second challenge with DEN2-16681 virus.
[c]Pre-challenge sera were obtained from the monkeys on day 240 after primary immunization.
+ = positive viremia by RT - PCR.
- = negative viremia by RT - PCR.

Table 4
Flavivirus neutralizing antibodies in monkey sera before and after challenge with DEN2-16681 virus.

Immunization	Monkeys	Reciprocal neutralizing antibodies (50%)[a]														
		Pre-challenge[c]					Day 30[c]					Day 60				
		DEN1	DEN2	DEN3	DEN4	JE	DEN1	DEN2	DEN3	DEN4	JE	DEN1	DEN2	DEN3	DEN4	JE
DEN2/1-EP	AF152	640	10	40	-	-	1,280	1,280	640	-	20	1,280	1,280	640	10	10
	AF154	320	20	40	-	-	1,280	1,280	640	-	-	1,280	1,280	320	-	-
	AF181	640	160	20	-	-	320	-	80	-	10	320	-	20	-	-
DEN2/1-VP	AF143	80	-	10	-	-	160	-	20	-	-	80	-	20	-	-
	AF165	640	-	40	-	10	1,280	640	160	-	-	80	1,280	40	-	-
	AF170	160	-	40	10	-	1,280	640	640	0	0	1,280	640	320	-	-
BA-1	AF179	640	-	10	-	-	1,280	1,280	640	-	10	1,280	1,280	160	-	-
	AF188	640	160	40	-	10	1,280	160	80	-	-	1,280	160	160	-	-

[a]Dilution of serum which resulted in 50% reduction of the input virus PFU.
[b]Pre-challenge sera were obtained from the monkeys on day 240 after primary immunization with chimeric viruses.
[c]Days are time after the secondary challenge with DEN2-16681 virus.
- = lowest dilution of serum (1:10) resulted in less than 50% reduction of the input virus PFU.

Table 5
Level of IFN-γ and IL2-sRα in cynomolgus monkey sera after challenge with DEN2-16681 virus.

Immunization	Monkey	Level of IFN-γ (pg/ml)							Level of IL2-sRα (pg/ml)						
		Pre-challenge[a]	Day 3[b]	Day 5	Day 7	Day 9	Day11	Day 14	Pre-challenge	Day 3	Day 5	Day 7	Day 9	Day 11	Day 14
DEN2/1-EP	AF152	-	-	-	-	-	-	-	140	205	310	205	180	180	180
	AF154	12	36	140	4	-	-	-	260	290	280	260	410	310	280
	AF181	-	-	-	-	-	-	-	380	200	410	540	380	320	370
DEN2/1-VP	AF143	-	-	-	-	-	-	-	210	210	280	280	310	260	260
	AF165	-	-	-	-	-	-	-	160	140	120	120	180	100	210
	AF170	-	-	-	-	-	-	-	490	530	600	620	635	450	660
BA-1	AF179	-	-	-	-	-	-	-	415	500	530	190	415	330	310
	AF188	-	-	-	-	-	-	-	1,050	1,070	850	1,020	990	780	820

[a]Pre-challenge sera were obtained from the monkeys at day 240.
[b]Days are time after the secondary challenge with DEN2-16681 virus.
- = level below 4 pg/ml.

virus.

Several chimeric flaviviruses have had their potential for use as vaccines investigated (Pletnev *et al*, 1992, 1993; Putnak *et al*, 1995; Bray *et al*, 1996, Men *et al*, 1996; Lai *et al*, 1998; Huang *et al*, 2000). Infectious clones of yellow fever (YF) virus, strain 17D (Chambers *et al*, 1999; Guirakhoo *et al*, 1999), and DEN2-PDK53 (Kinney *et al*, 1997) were developed from vaccine (YF) or candidate vaccine (DEN2-PDK53) viruses that have been tested in humans. Chimeric YF-17D/DEN2 and YF-17D/JE viruses containing the prM/E gene region of DEN2 and JE virus respectively have been successfully tested in non-human primates (Chambers *et al*, 1999; Guirakhoo *et al*, 1999; 2000). There are 32 amino acid differences, including 12 substitutions in the E protein gene, between the parental YF Asibi virus and its 17D vaccine derivative (Hahn *et al*, 1997). It is possible that chimeric YF-17D viruses that express structural genes of a virulent, wild-type flavivirus might not be attenuated if the mutations in the E gene of YF 17D virus are the major determinants of attenuation in that vaccine virus. Unlike the YF17D virus, the genetic determinants of attenuation of the DEN2-PDK53 virus have been mapped and are located in the nonstructural gene regions of the viral genome (Butrapet *et al*, 2000). Flavivirus chimeras based on the attenuated genotype of DEN2-PDK53 virus and expressing attenuated or wild-type structural genes of other flaviviruses might result in attenuated chimeric viruses such as our DEN2/1-EP and DEN2/1-VP viruses. The DEN2-PDK53 backbone appears to be a good candidate for the vectoring of live attenuated flavivirus vaccines. Chimeric DEN2/1 virus might be an effective component of a tetravalent vaccine that contains DEN2-PDK53 virus and chimeric DEN2/1, -2/3, and -2/4 viruses, all of which share common nonstructural genes of the PDK53 virus. Such a tetravalent vaccine might avoid or reduce viral interference vaccinees (Huang *et al*, 2000). Because the attenuation loci of the chimeric tetravalent vaccine reside on the same PDK53 nonstructural backbone, any potential recombination among the different chimeric vaccine viruses would not affect the attenuation.

Structural proteins and non-structural proteins of DEN virus are known to be targets for CTL-mediated immune responses. The structural proteins induce serotype-specific CTL responses, while nonstructural protein 3 (NS3) induces both serotype-specific and serotype-cross-reactive CTL (Halstead, 1988; Livingston *et al*, 1995; Rothman *et al*, 1996; Spaulding *et al*, 1999). Activation of serotype-cross-reactive CTL during secondary infection with DEN viruses may contribute to the pathogenesis of DHF by stimulating over-production of cytokines and increased cytolytic activity (Kurane *et al*, 1994; Rothman *et al*, 1996; Spaulding *et al*, 1999). The levels of T-cell activation markers, such as IFN-γ, IL-2, sCD4, sCD8, and IL2-sRα, are significantly elevated in the sera of patients with DHF/DSS (Kurane *et al*, 1991). CTL induced by infection with DEN1, DEN3, or DEN4 virus may cross-react with T-cell epitopes presented during a secondary infection with DEN2 virus to greater extent than occurs in the reverse situation involving primary infection with DEN2 virus and secondary infection with a heterologous DEN1, DEN3, or DEN4 virus (Spaulding *et al*, 1999). It has been reported that DEN2-PDK53 virus induces predominantly DEN2 serotype-specific CTL responses in vaccinees (Dharakul *et al*, 1994). The strategy of immunization with chimeric viruses which all contain the DEN2-PDK53 virus-specific nonstructural gene backbone may avoid DHF/DSS following infection with other serotypes of virus. In the present study, our results showed that primary immunization with chimeric DEN2/1 virus, then challenge with DEN1-16007 virus followed by a second virus challenge with DEN2-16681 virus, did not cause increased levels of IFN-γ in the sera of the immunized monkeys, except for monkey AF154. Slight increases in IL2-sRα levels indicated activation of T-cell function after virus challenge. Monkeys immunized with DEN2/1 virus appeared to be protected from sequential challenge with DEN1 and DEN2 viruses. Our findings here support the hypothesis that CTL induced by DEN2-PDK53-based chimeric viruses may not efficiently recognize heterolo-

gous serotypes of dengue virus. This property might help to avoid the immunopathogenesis of DHF/DSS in vaccinees who later become infected with wild-type DEN viruses. DEN2-PDK53 virus and chimeric, DEN2-PDK53-based viruses are potentially effective components of a live-attenuated, broadly immunogenic, tetravalent DEN vaccine that may permit equivalent replication of each vaccine component with decreased viral interference, decreased potential problems resulting from possible recombination events, and decreased potential for immunopathogenesis.

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