



Protection against dengue virus by non-replicating and live attenuated vaccines used together in a prime boost vaccination strategy

Monika Simmons^{a,*}, Timothy Burgess^a, Julia Lynch^b, Robert Putnak^b

^a Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, MD 20910, USA

^b Division of Viral Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA

ARTICLE INFO

Article history:

Received 11 August 2009

Returned to author for revision

8 September 2009

Accepted 16 October 2009

Available online 13 November 2009

Keywords:

Dengue DNA

Purified inactivated virus

Live attenuated virus

Prime boost

ABSTRACT

A new vaccination strategy for dengue virus (DENV) was evaluated in rhesus macaques by priming with tetravalent purified inactivated virus (TPIV) or tetravalent plasmid DNA vaccines expressing the structural prME gene region (TDNA) then boosting 2 months later with a tetravalent live attenuated virus (TLAV) vaccine. Both vaccine combinations elicited virus neutralizing (N) antibodies. The TPIV/TLAV combination afforded complete protection against DENV 3 challenge at month 8. In a second experiment, priming with TPIV elicited N antibodies against all four serotypes (GMT 1:28 to 1:43). Boosting with TLAV led to an increase in the GMT for each serotype (1:500 to 1:1200 for DENVs 1, 3, and 4, and greater than 1:6000 for DENV 2), which declined by month 8 (GMT 1:62 for DENV 3, 1:154 for DENV 1, 1:174 for DENV 4, and 1:767 for DENV 2). After challenge with each one of the four DENV serotypes, vaccinated animals exhibited no viremia but showed anamnestic antibody responses to the challenge viruses.

Published by Elsevier Inc.

Introduction

The dengue viruses (DENVs), comprised of four distinct serotypes, DENVs 1, 2, 3, and 4, in the family Flaviviridae, are transmitted to humans by *Aedes aegypti* mosquitoes resulting in an estimated 100 million infections annually in the tropics and subtropics (Monath, 1986). Most dengue infections are asymptomatic or result in uncomplicated dengue fever (DF), a mild to moderate acute febrile illness. Because immunity following infection is serotype specific, individuals remain at risk for secondary infection from other serotypes. A small but significant number of individuals develop dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are severe, sometimes life-threatening illnesses. The risk for DHF and DSS is greater following a second dengue infection (Halstead, 1988), although the pathogenesis is not fully understood. While serotype-specific virus neutralizing antibodies directed against the virion envelope (E) antigen are associated with protection (Kaufman et al., 1987), non-neutralizing, cross-reactive antibodies appear to mediate the infection of Fc receptor bearing cells, e.g., monocytes and macrophages, leading to higher viremia and more severe disease (Halstead, 1988) underscoring the need for a vaccine that protects against all four DENV serotypes.

Most recent efforts to develop a dengue vaccine have focused on live attenuated viruses (LAVs) (Bhamarapravati, Yoksan, 1997).

However, the safety and efficacy of these products has yet to be demonstrated (Sabchareon et al., 2002). Unpredictable interactions among the four virus serotypes in tetravalent LAV (TLAV) formulations have historically made it difficult to achieve a vaccine that is both satisfactorily attenuated and highly immunogenic. This was demonstrated by significant dengue-like illness seen in clinical trials of one candidate dengue TLAV vaccine where an under-attenuated DENV 3 component in the tetravalent formulation was the apparent cause of the reactogenicity (Kitchener et al., 2006), which effectively halted the further clinical development of that product.

Three other TLAV vaccine candidates are currently in clinical trials. One of these vaccines is attenuated by serial passage of wild-type viruses in PDK cells followed by production in fetal rhesus lung (FRhL) diploid cells (Halstead and Marchette, 2003; Eckels et al., 2003; Sun et al., 2003; Sun et al., 2009). The other two are genetically engineered chimeras, with the dengue structural genes expressed from an infectious clone of yellow fever virus 17D vaccine (Gurakhoo et al., 2006), or an infectious clone of DENV 4 containing attenuating mutations in the 3' non-coding region of the viral genome (McArthur et al., 2008; Durbin et al., 2006a,b), produced in Vero cells. While all three of these products have good safety profiles, it has proven difficult to achieve tetravalent virus neutralizing antibody responses after one dose of vaccine, possibly because some virus serotypes replicate more efficiently so as to be dominant over or interfere with other serotypes in the tetravalent mixture. Evidence for cross serotype interference can also be seen in rhesus monkeys (Guy et al., 2009). Although serotype dominance or interference is less apparent after booster immunizations, the need for multiple doses and long immunization schedules limit the suitability of these products as

* Corresponding author. Fax: +1 301 319 7451.

E-mail addresses: monika.simmons@med.navy.mil (M. Simmons), timothy.burgess2@med.navy.mil (T. Burgess), julia.lynn@us.army.mil (J. Lynch), joseph.robert.putnak@us.army.mil (R. Putnak).

Report Documentation Page			Form Approved OMB No. 0704-0188		
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE SEP 2009		2. REPORT TYPE		3. DATES COVERED 00-00-2009 to 00-00-2009	
4. TITLE AND SUBTITLE Protection against dengue virus by non-replicating and live attenuated vaccines used together in a prime boost vaccination strategy			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S)			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Medical Research Center,Viral and Rickettsial Diseases Department,Silver Spring,MD,20910			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT A new vaccination strategy for dengue virus (DENV) was evaluated in rhesus macaques by priming with tetravalent purified inactivated virus (TPIV) or tetravalent plasmid DNA vaccines expressing the structural prME gene region (TDNA) then boosting 2 months later with a tetravalent live attenuated virus (TLAV) vaccine. Both vaccine combinations elicited virus neutralizing (N) antibodies. The TPIV/TLAV combination afforded complete protection against DENV 3 challenge at month 8. In a second experiment, priming with TPIV elicited N antibodies against all four serotypes (GMT 1:28 to 1:43). Boosting with TLAV led to an increase in the GMT for each serotype (1:500 to 1:1200 for DENVs 1, 3, and 4, and greater than 1:6000 for DENV 2), which declined by month 8 (GMT 1:62 for DENV 3, 1:154 for DENV 1, 1:174 for DENV 4, and 1:767 for DENV 2). After challenge with each one of the four DENV serotypes, vaccinated animals exhibited no viremia but showed anamnestic antibody responses to the challenge viruses.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

traveler's vaccines. Interestingly, individuals with prior immunity to non-dengue flaviviruses Japanese encephalitis or yellow fever were shown to demonstrate secondary type immune responses after primary vaccination with DEN LAV vaccines. (Kanesa-Thanas et al., 2003). An earlier monovalent DENV 2 LAV vaccine candidate was also found to be more immunogenic in yellow fever immune subjects (Scott et al., 1983), probably due to increased vaccine viremia (Bancroft et al., 1981). This suggests that immunological priming, even with heterologous antigen, can significantly alter the host's immune responses to DEN LAV vaccines.

In addition to the TLAV vaccines currently in advanced clinical trials, there are other DENV vaccine candidates in earlier stages of testing, including a chimeric LAV vaccine in an attenuated DENV 2 background (Huang et al., 2003), a purified inactivated whole virus (PIV) vaccine (Putnak et al., 2005), a recombinant subunit vaccine based on the aminoterminal 80% of E protein (Putnak et al., 2005), a recombinant subunit vaccine based on E domain III (Simmons et al., 2001), and a DNA vaccine expressing the prME structural gene region (Simmons et al., 2001). One advantage of non-replicating vaccines is that they might immunize more quickly and exhibit less serotype dominance or interference than LAV vaccines, although some might be less effective at eliciting cell-mediated immune responses and at conferring long-term immunity. Alternatively, it might be possible to use some combination of live and non-replicating vaccines to achieve a more balanced immune response in a shorter period of time. The idea of priming and boosting with different vaccines has previously been tested experimentally for immunizing against HIV (Barnett et al., 1997) and malaria (Sedegah et al., 1998).

In the present study, we tested a prime boost approach for dengue in rhesus macaques by priming the animals with either TPIV or TDNA vaccines followed by boosting with a TLAV vaccine. The vaccinated animals were then challenged with near wild-type strains of DENV to determine if they were protected. Although dengue infection in the rhesus model is clinically inapparent, dengue non-immune animals become viremic when challenged; therefore, in this model viremia serves as a surrogate for human infection, and as a way for measuring the protective efficacy of DENV vaccine candidates (Sun et al., 2006). We found that animals primed with non-replicating vaccines developed mainly low titered virus neutralizing antibodies and also non-neutralizing antibodies, which could be measured by ELISA. After boosting with the TLAV vaccine, the animals exhibited a secondary type immune response with higher titered and more broadly cross serotype-reactive neutralizing antibodies than were seen after priming or after a single dose of TLAV vaccine. Animals that received the TPIV/TLAV prime boost vaccine were completely protected against viremia after virus challenge. These results suggest that a prime boost vaccination strategy might be effective for protecting humans against dengue.

Results

Non-replicating tetravalent DENV vaccines primed rhesus macaques for an immune response to a tetravalent live attenuated virus (TLAV) vaccine.

An initial experiment was performed in 16 flavivirus naïve rhesus macaques to determine the most effective priming vaccine, dengue tetravalent DNA (TDNA) or tetravalent PIV (TPIV) vaccine. Animals were randomly assigned to one of four groups with each group containing 4 animals, and inoculated with either two doses of TDNA vaccine (Group 1), one dose of TPIV vaccine (Group 2), or for comparison, with one dose of TLAV vaccine (Group 3). An unvaccinated control group received phosphate-buffered saline only (Group 4). Two months later, all animals except the saline controls were boosted with one dose of the TLAV vaccine. Measurement of virus neutralizing (N) and total antibody titers against all four DENV serotypes after vaccination and measurement of serum viremia after

monovalent challenge with a near wild-type strain of DENV 3 were used to evaluate the more effective prime boost vaccine combination.

Antibody responses to vaccination

After primary vaccination, all groups except the saline control made antibodies to all four DENV serotypes measured by ELISA (data not shown) and virus plaque reduction neutralization test (PRNT50) (Fig. 1) and remained positive for virus N antibodies 30 and 60 days after primary vaccination. The N antibody geometric mean titers (GMTs) for serotype 2 were higher than for the other serotypes. After boosting with the TLAV vaccine, the N antibody GMTs for all serotypes increased in all groups, reaching a peak 1 month later, at which time the highest N antibody GMTs were against DENV 2 and DENV 4. The N

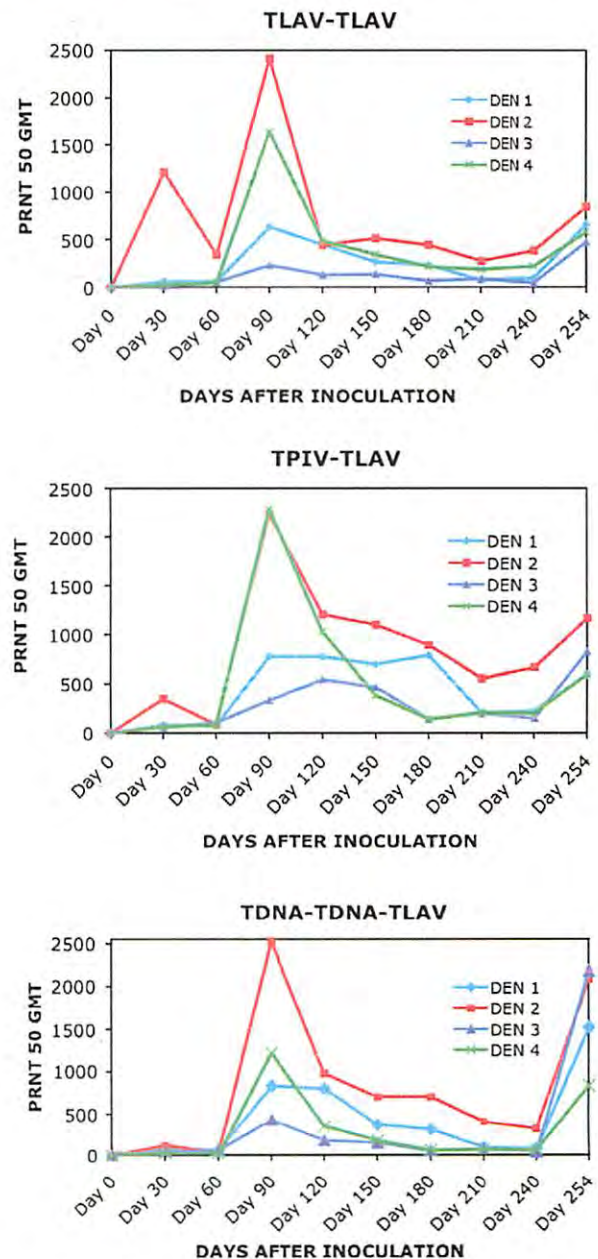


Fig. 1. DENV neutralizing antibody responses in rhesus macaques vaccinated with combinations of tetravalent live attenuated virus (TLAV), tetravalent purified inactivated virus (TPIV), and tetravalent DNA (TDNA) vaccines and then challenged at month 8 with live, non-attenuated DENV 3. Reciprocal geometric mean neutralizing antibody PRNT50 titers are presented for all time points.

antibody titers then declined sharply over the next 2 months and more gradually thereafter until administration of the challenge virus at month 8. On the day of challenge, all groups remained antibody positive, with Group 2 (TPIV/TLAV) exhibiting the highest total and N antibody titers and with the highest N antibody titers being against DENV 2.

Responses to monovalent wild-type DENV 3 challenge

To assess protection, a near wild-type isolate of DENV 3 was chosen because the lowest virus N antibody titers in all groups were against serotype 3. The DENV 3 challenge virus was administered to all animals including the saline controls, and sera were collected for 10 consecutive days for measuring viremia. The post-challenge viremia results are shown in Table 1. Group 4 (saline) exhibited on average 5 days of viremia, whereas Group 1 (TDNA/TDNA/TLAV) exhibited 1.5 days of viremia on average and Group 2 (TPIV/TLAV) and Group 3 (TLAV/TLAV) exhibited no viremia after the virus challenge. In this experiment, there was a correlation between higher total anti-DENV 3 Ig titers on the day of challenge measured by ELISA and protection (Fig. 2). After the virus challenge, N antibody GMTs to all serotypes increased in all vaccine groups, with a 50-fold increase in anti-DENV 3 N antibody titer for the animals in Group 1 (TDNA/TLAV), a 5-fold increase for the animals in Group 2 (TPIV/TLAV), and a 10-fold increase for the animals in Group 3 (TLAV/TLAV) (Fig. 1). Based on these results, a second experiment was performed to determine whether priming with a TPIV vaccine followed by boosting with a TLAV vaccine can confer protection against all four DENV serotypes.

DENV TPIV and DENV TLAV vaccines used in a prime boost vaccine combination protected rhesus macaques against challenge with all four DENV serotypes

For this experiment, 32 flavivirus naïve animals were randomly assigned to two cohorts of 16 animals each. The first cohort was vaccinated with TPIV vaccine followed 2 months later by TLAV

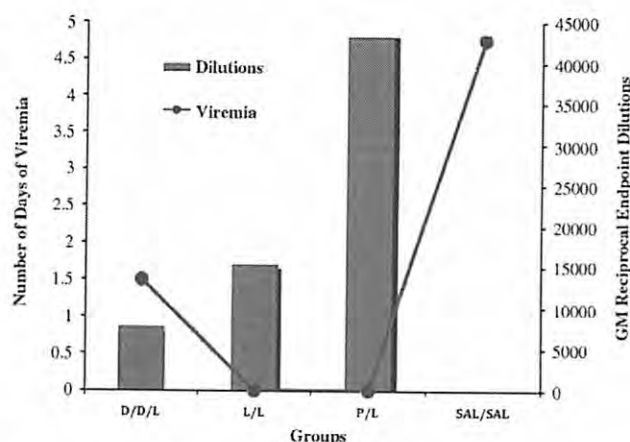


Fig. 2. Anti-DENV 3 IgG antibody responses measured by ELISA on the day of challenge and protection from viremia after challenge. Bars indicate reciprocal geometric mean endpoint titers (GMT) measured by ELISA (≥ 0.1 OD), and circles indicate mean days of viremia measured by virus amplification in Vero cells followed by IFA.

vaccine. The second (control) cohort was mock vaccinated with phosphate-buffered saline. At month 8, both the vaccine and the control cohorts were challenged with low passage, near wild-type DENV viruses, with 4 vaccinated and four control animals per group receiving either DENV 1, 2, 3, or 4, one challenge virus administered per group. Sera were collected at intervals to measure antibody responses to vaccination and challenge, and daily for 10 days after both TLAV vaccination and virus challenge to measure viremia.

Antibody responses after vaccination with TPIV and boosting with TLAV vaccine

The results in Fig. 3 show that after primary vaccination with TPIV, all animals had N antibodies to all four DENV serotypes. Although the GMTs were low they were similar for all serotypes.

Table 1

Viremia in rhesus macaques immunized with combinations of tetravalent live attenuated virus (TLAV), tetravalent purified inactivated virus (TPIV), and tetravalent DNA (TDNA) vaccines and then challenged with live, non-attenuated DENV 3.

Vaccine combination	Monkey ID	Days viremia detected*										Mean days of viremia
		1	2	3	4	5	6	7	8	9	10	
TLAV/TLAV	P146											0
	860Z											
	3158											
	928Z											
TPIV/TLAV	890Z											0
	A63Z											
	916Z											
	A96Z											
TDNA/TDNA/TLAV	A71											1.5
	856Z											
	894Z											
	922Z											
SALINE/SALINE	914Z											4.75
	B85											
	868Z											
	898Z											

*Filled black squares show the days after challenge that dengue virus was detected in sera by amplification in Vero cells followed by detection of virus-infected cells using IFA.

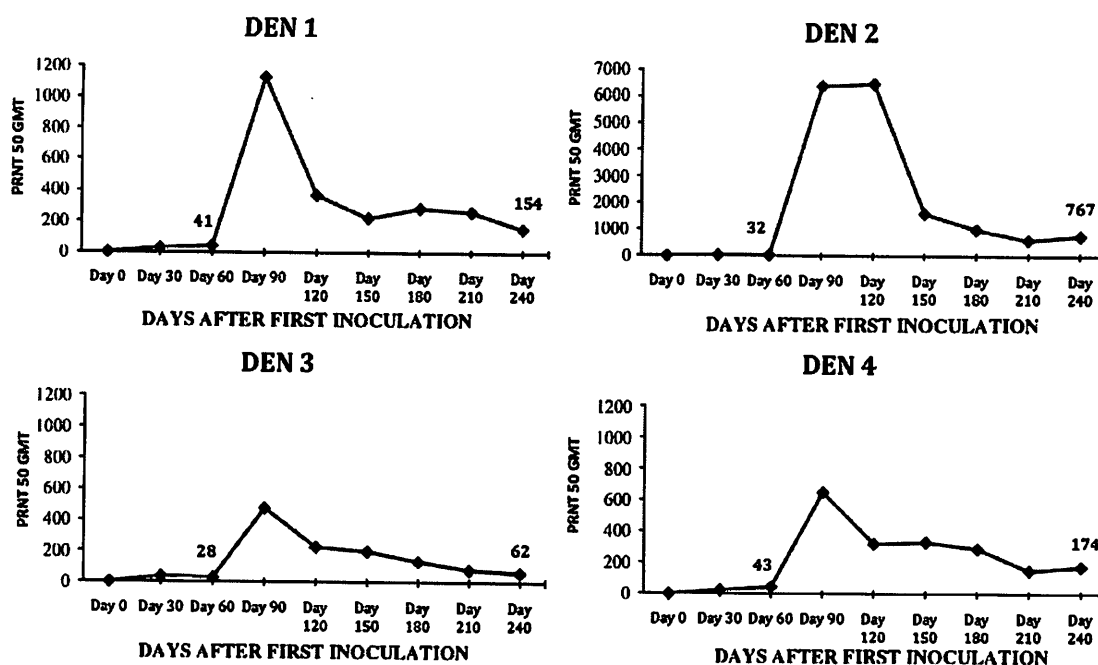


Fig. 3. DENV neutralizing antibody responses in rhesus macaques primed with tetravalent purified inactivated virus (TPIV) vaccine (Day 0), boosted with tetravalent live attenuated virus (TLAV) vaccine (Day 60), and then challenged at month 8 with live, non-attenuated DENV 1, DENV 2, DENV 3, or DENV 4. Reciprocal geometric mean neutralizing antibody PRNT50 titers are presented from day 0 until the day of challenge, day 240.

The N antibody GMTs then increased after the TLAV booster vaccination given at month 2 and peaked between study day 90 and day 120, with the highest GMT achieved for DENV 2 (1:6000) and lower GMTs ranging from 1:500 to 1:1200 for the other serotypes. The GMTs for all serotypes then declined at variable rates, with the steepest decline in titer occurring within one to 2 months after peaking, followed by a more gradual decline to the day of challenge, similar to what was seen in the first experiment. On the day of challenge (month 8), the N antibody GMTs were 1:62 for serotype 3, 1:154 for serotype 1, 1:174 for serotype 4, and 1:767 for serotype 2.

Viremia responses after TLAV vaccination and tetravalent wild-type DENV challenge

While priming with TPIV vaccine did lead to better immune responses to the TLAV vaccine, it did not enhance the replication of any of the vaccine viruses leading to detectable viremia (data not shown). In order to determine if the vaccinated animals were protected against all four DENV serotypes, both vaccinated and control (mock vaccinated) cohorts were challenged with near wild-type challenge viruses, with groups of 4 vaccinated and 4 control animals each receiving a different challenge virus, DENV 1, 2, 3, or 4. The post-challenge viremia results are shown in Table 2. In the mock (PBS) vaccinated cohort, all animals challenged with each DENV serotype became viremic, thus demonstrating a successful challenge for each serotype. The animals challenged with DENV 4 exhibited the shortest duration of viremia with a mean of 2.75 days, whereas the animals challenged with DENV 1, DENV 2, or DENV 3 exhibited 4 to 5 mean days of viremia, consistent with previous rhesus challenge experiments (Simmons and Putnak, unpublished results). In contrast, in the vaccinated cohort, there was no detectable viremia with any of the challenge virus serotypes. There were, however, anamnestic antibody responses observed in most vaccinated animals after the virus challenge, with increases in N antibody titers against all serotypes and an increase in total antibody titers against DENV 2, DENV 3, and DENV 4 (Fig. 4).

Discussion

It has proven very difficult and costly to develop tetravalent dengue vaccine candidates that immunize and confer both immediate and long-term immunity to all four virus serotypes with a minimum of vaccine-associated adverse effects. Following primary vaccination of flavivirus naïve subjects in clinical trials with some tetravalent live attenuated virus (TLAV) vaccine formulations, protective virus neutralizing antibodies against some serotypes are very low or undetectable, suggesting that those serotypes replicate and immunize poorly. In contrast, other virus serotypes appear to dominate the immune response and sometimes cause viremia, which may lead to increased vaccine reactogenicity seen as dengue-like illness (Kitchener et al., 2006). Although a second dose of TLAV vaccine generally leads to improved tetravalent seroconversion rates and higher virus neutralizing antibody titers with reduced reactogenicity, it appears that this must be administered 6 months or longer following primary vaccination in order to be most effective. Non-replicating tetravalent dengue vaccines may exhibit less serotype dominance and vaccine-associated adverse effects and allow for the use of shorter time intervals between primary and booster vaccinations. However, such vaccines may not be as effective as TLAV vaccines at conferring long-term immunity. Therefore, in this study an alternative strategy for vaccinating against dengue was tested in the rhesus macaque animal model. This involved priming the animals with a non-replicating vaccine, either a tetravalent purified inactivated virus (TPIV) or a tetravalent plasmid DNAs expressing the prME structural gene region (TDNA), followed by boosting with a tetravalent live attenuated virus (TLAV) vaccine. The aims of this study were threefold: (i) to demonstrate that priming with a non-replicating vaccine followed by boosting with a TLAV vaccine is able to elicit high titered virus neutralizing (N) antibodies and confer protection against all four DENV serotypes; (ii) to demonstrate that a heterologous prime boost vaccination strategy has a low risk for eliciting vaccine-related adverse events and might even reduce the risk for reactogenicity by moderating the replication of under-attenuated viruses in a tetravalent LAV vaccine; and (iii) to demonstrate the feasibility of a compressed immunization schedule (2–3 months vs. 6–7 months),

Table 2

Viremia in rhesus macaques immunized with tetravalent purified inactivated virus (TPIV) vaccine, boosted with tetravalent live attenuated virus (TLAV) vaccine, and then challenged with live, non-attenuated strains of DENV 1, 2, 3, or 4.

Challenge virus	Vaccine	Monkey ID	Days viremia detected*										Group mean days of viremia
			1	2	3	4	5	6	7	8	9	10	
DENV 1	TPIV/TLAV	CX5E											0
		CX5A											
		CT3R											
		DA96											
	SALINE	97D439											4.5
		DA88											
		DA2W											
		CX5F											
DENV 2	TPIV/TLAV	CV9L											0
		DB10											
		DA83											
		DA2V											
	SALINE	CX53											5.0
		DH4X											
		DA9K											
		M001											
DENV 3	TPIV/TLAV	DP80											0
		DA97											
		DA91											
		M007											
	SALINE	DA93											4.25
		CX49											
		DA9J											
		DR45											
DENV 4	TPIV/TLAV	97C213											0
		CX5V											
		DA9V											
		DA9T											
	SALINE	DR31											2.75
		DA64											
		DA9G											
		CX5G											

*Filled black squares show the days after challenge that dengue virus was detected in sera by amplification in Vero 81 cells followed by detection of virus-infected cells by IFA.

potentially allowing for more rapid development of protective immunity than can be achieved with existing TLAV vaccine candidates.

An initial experiment was performed in rhesus macaques to determine whether TPIV or TDNA vaccine was more effective at priming for a booster response to a TLAV vaccine administered 2 months later. After priming with two doses of TDNA, one dose of TPIV, or one dose of TLAV vaccine, the seroconversion rates and virus N antibody titers against each serotype measured by PRNT were comparable among groups, except for much higher DENV 2 N antibody titers in the TLAV group. Following boosting with the TLAV vaccine, the kinetics of the anamnestic N antibody response was similar for all groups. The highest N antibody GMTs were against DENV 2 followed by DENVs 4, 1, and 3. Measurement of total DENV 3 IgG titers by ELISA on the day of challenge showed that the TPIV/TLAV group had the highest titer followed by the TLAV/TLAV group, while the TDNA/TDNA/TLAV group had the lowest total IgG titer. Because

the DENV 3 N antibody titers were lower than those for the other serotypes in all three immunization regimens, this virus was chosen for the challenge.

After DENV 3 challenge, complete protection against viremia was seen in groups that received the TPIV/TLAV and TLAV/TLAV combinations. Breakthrough viremia was detected in the TDNA/TDNA/TLAV group, although it was reduced in duration compared to the mock-vaccinated controls. After the DENV 3 challenge, all groups including the TLAV/TLAV group exhibited anamnestic N antibody increases for all serotypes. Not surprisingly, the greatest increase in N antibody titer after challenge was seen in the TDNA/TDNA/TLAV group, with the greatest fold increase in titer and the highest absolute titer against DENV 3, consistent with the breakthrough viremia observed in those animals. It is unclear why the TDNA/TDNA/TLAV combination provided incomplete protection from challenge. Priming by TDNA vaccine required two doses vs. one dose for TPIV. It is possible that at least some epitopes important for protection were

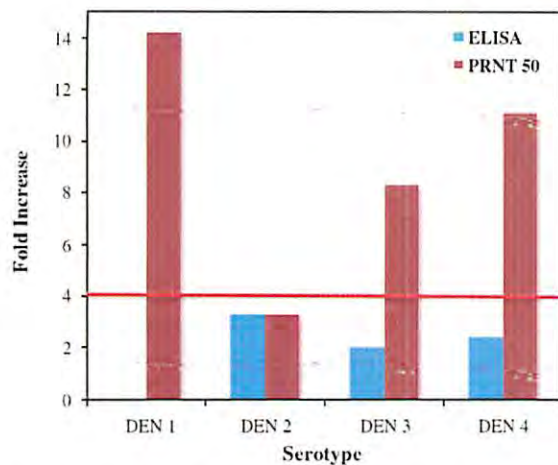


Fig. 4. Anamnestic anti-DENV antibody responses in vaccinated rhesus macaques measured by ELISA and PRNT using sera collected 2 weeks after tetravalent DENV challenge. Blue bars indicate reciprocal geometric mean endpoint titers (GMT) measured by ELISA (≥ 0.1 OD), and brown bars indicate reciprocal geometric mean 50% plaque reduction neutralizing antibody titers (GMT PRNT50) measured by PRNT. An anamnestic antibody response was defined as ≥ 4 -fold increase in titer.

either not correctly expressed or presented by this vaccine. Somewhat surprisingly, the best correlate of protection against viremia in this experiment was the total anti-DENV 3 IgG antibody measured by ELISA on the day of challenge, rather than the neutralizing antibody titer.

Based on results from the first experiment, the TPIV/TLAV prime boost vaccine combination was tested for the ability to confer protection against viremia after challenge with wild-type strains of all four DENV serotypes. Similar to the first experiment, animals were primed with one dose of TPIV, boosted with TLAV 2 months later, and then challenged at month 8, with four animals receiving each challenge virus serotype. After the administration of the TPIV vaccine, the animals developed low but measurable N antibody titers against each serotype demonstrating successful priming. After the TLAV booster, the N antibody titers against each serotype increased more than 10-fold, indicating strong anamnestic antibody responses. Importantly, after the TLAV booster no vaccine-associated viremia was detected for any serotype nor did the animals show any other adverse effects, suggesting that TPIV priming was safe. N antibody titers declined sharply at first and then more gradually to the day of challenge (month 8) at which time the highest N antibody titers were against DENV 2 (1:767) followed by DENV 4 (1:174), DENV 1 (1:154), and DENV 3 (1:62). In comparison, the total DENV IgG titers on the day of challenge were highest for DENV 1 and DENV 2, followed by DENV 4 and DENV 3. This difference between N and total antibody titers among serotypes may have been due to the greater serotype cross-reactivity of total IgG antibodies compared with N antibodies, which tend to be more serotype specific. After the virus challenge, there was complete protection against viremia from all four serotypes. There were, however, strong anamnestic N antibody responses to three of the four challenge viruses as shown in Fig. 3, with approximately 8- to 14-fold increases in N antibody titers for DENVs 3, 4, and 1 but only about a threefold increase in titer for DENV 2, possibly indicating near sterile immunity to DENV 2. In comparison, total antibody titers, which were higher prior to challenge, did not increase as much after challenge, with only two- to threefold increases for DENVs 2, 3, and 4 and no measurable increase for DENV 1.

These two experiments clearly demonstrated that rhesus macaques could be successfully immunized and protected against all four DENV serotypes using TPIV vaccine followed by TLAV vaccine in a prime boost vaccination strategy. Low but measurable titers of N

antibody were elicited against each DENV serotype by primary vaccination with TPIV demonstrating successful priming. However, the presence of these virus N antibodies after priming apparently did not inhibit replication of the TLAV vaccine enough to prevent successful boosting, as evidenced by strong anamnestic N antibody responses following TLAV administration. Although it could not be demonstrated in these experiments, it is possible that low titered virus neutralizing antibodies may be able to modulate the replication of under-attenuated strains present in TLAV vaccine formulations, thereby reducing vaccine reactogenicity. It is also notable that non-neutralizing, presumably serotype cross-reactive antibodies generated in response to primary vaccination did not enhance the replication of the attenuated vaccine viruses to the point of viremia.

Although the significance of the immune-dominance of DENV serotype 2 in terms of the N antibody response is unclear, the uniform development of N antibodies against all four DENV serotypes within 1 month after the TLAV booster, without evidence for vaccine-induced viremia, further demonstrated that priming with non-replicating vaccine was effective and suggests that protective immunity can be achieved safely and rapidly by heterologous prime boost vaccination. Arguably, however, TLAV vaccine alone, when two doses were administered 2 months apart, was also able in a short period of time to elicit moderate to high titered N antibodies against all four DENV serotypes in the rhesus model, in contrast to the requirement for a longer time interval between doses for effective immunization of humans. This result suggests some shortcomings to the use of rhesus macaques for predicting the outcome of vaccination in humans. Nevertheless, viremia in rhesus macaques is the best animal model for dengue infection currently available, and the results clearly demonstrate that animals vaccinated with TPIV followed by TLAV vaccine were completely protected against viremia after challenge 8 months later with near wild-type DENV strains representing each serotype.

The reason for the challenge breakthrough seen in the first experiment with DENV 3 viremia in animals vaccinated with the TDNA/TDNA/TLAV combination is unclear. The low DENV 3 N antibody GMT (1:41) at the time of challenge might have been a plausible explanation except that the TLAV/TLAV group had a similarly low DENV 3 GMT (1:47), yet that group was completely protected. Interestingly, the animals in the TDNA/TDNA/TLAV group also had the lowest total DENV IgG antibody titers at month 8, and there was a correlation between total DENV IgG titers at the time of challenge and protection against viremia. Therefore, it is possible that non-neutralizing antibodies may also play an important role in protective immunity, perhaps through complement mediated lysis of virus and virus-infected cells, or antibody-dependent cell-mediated cytotoxicity (ADCC), which is difficult to measure in vitro.

In future studies, it will be important to further define the correlates of immunity to DENV as well as test the safety and immunogenicity of heterologous prime boost vaccine approaches in a Phase 1 clinical trial.

Materials and methods

Viruses

Cell culture supernatant harvested from Vero cells infected with DENV 1 (West Pac 74), DENV 2 (S16803), DENV 3 (CH53489), and DENV 4 (341750) was used as virus stock for the plaque reduction neutralization test (PRNT) and to prepare antigen for the ELISA.

Plaque-reduction neutralization assay

Plaque-reduction neutralization tests (PRNTs) were performed to measure DENV neutralizing antibodies, using a method modified from that originally described (Russell et al., 1967). Vero cell monolayers were seeded in six-well plates (Falcon; Becton Dickinson, Lincoln

Park, NJ) and incubated at 37 °C in a CO₂ incubator. Sera from immunized rhesus macaques were tested using serial twofold dilutions starting at 1:10 to 1:640 or as required to reach an endpoint. The serum dilutions were mixed with each parental DENV serotype to obtain approximately 50 plaque forming units (PFU) per 0.2 ml, incubated at 37 °C for 30 min, then inoculated onto duplicate wells overlaid with nutrient agarose (EMEM, 2% FBS, 1% agarose). Plaques were visualized on day 6 by staining with 0.02% neutral red in Hank's balanced salt solution. The number of plaques reported for each serum dilution was the average of the duplicate wells. The percent reduction in plaques was calculated by comparison of the results obtained with control sera from unimmunized rhesus macaques. The neutralization titer was the test serum dilution at which 50% plaque reduction occurred (PRNT₅₀ titer) determined by probit analysis.

Enzyme-linked immunosorbent assay

Antigen was prepared by centrifugation (27,000 rpm at 20 °C) of DENV-infected and -uninfected Vero cells for 2 h. Pellets were resuspended in PBS and pelleted again in 10% glycerol at 32,000 rpm for 2 h at 20 °C. Purified virions and control antigen were resuspended in PBS and stored at –20 °C until used. The analysis of sera from immunized rhesus macaques for DENV-2 antibodies was carried out as previously described (Simmons et al., 2006). Briefly, microtiter plate wells were coated with purified DENV 2 virions in PBS at 4 °C overnight followed by blocking with 5% non-fat dry milk in PBS/0.01% Tween 20 for 1 h at 37 °C. Plates were then incubated with the test sera at twofold dilutions starting at 1:100 in blocking buffer for 1 h at 37 °C. The secondary antibody was peroxidase-conjugated goat anti-human IgG (Kirkegaard & Perry, Gaithersburg, MD) diluted in blocking solution and incubated for 1 h at 37 °C. The 2,2'-azino di [3-ethyl benzthiazoline sulfonate (6)] (ABTS) peroxidase substrate system (Kirkegaard & Perry) was used to visualize dengue virus-specific antibody. Assays were performed in duplicate with a positive and negative control on every plate. The net optical density (OD) values were determined by subtracting the absorbance of test serum with negative control antigen from the absorbance of test serum with the DENV antigen. Endpoint dilution titers were determined by the dilution at which the OD value was at ≥ 0.10 .

Preparation and administration of live attenuated virus (LAV) vaccines

Production and testing of LAV vaccine candidates have previously been described in detail (Eckels et al., 2003; Sun et al., 2003, 2009). Briefly, the viruses were isolated from viremic sera from clinical DEN cases and attenuated by serial passage in primary dog kidney (PDK) cells, 6 to 50 PDK passages depending on the virus serotype and the vaccine formulation (Halstead and Marchette, 2003). Following PDK passage, the vaccines were finished by three additional passages in diploid fetal rhesus lung-2 (FhRL 2) cells to produce the master and production seeds and the vaccines. The candidate monovalent vaccine viruses were tested individually and demonstrated to elicit significantly reduced viremia compared to the wild-type parental isolated when administered to rhesus monkeys (Eckels et al., 2003). The monovalent LAV vaccines were formulated with excipients, 50 µg/ml neomycin sulfate, 5.5% lactose, and 1.9% human serum albumin as stabilizer, bottled, freeze dried, and stored at –20 °C. Prior to immunizations, each monovalent vaccine was rehydrated with sterile water for injection, and syringe mixed to produce tetravalent formulations, then administered to the animals by subcutaneous (SC) inoculation into the loose skin of the upper back at a dose of 5 log₁₀ pfu per serotype.

The tetravalent LAV (TLAV) formulation used for the first rhesus experiment consisted of DENV 1 (West Pac 74, 45A25, PDK 20), DENV 2 (S16803, PDK 50), DENV 3 (CH53489, PDK 20), and DENV 4

(341750, PDK 6). The TLAV formulation for the second experiment was the same except that DENV 1 PDK 20 was replaced by DENV 1 PDK 27. This substitution was made necessary because the DENV 1 PDK 20 vaccine was re-derived by RNA transfection of FRhL cells and the re-derived vaccine was genetically less stable than the original DENV 1 PDK 20 vaccine used in the first experiment.

Preparation and administration of purified inactivated virus (PIV) vaccine

The TPIV vaccine was prepared from DENVs (DENV 1 West Pac 74, DENV 2 S16803, DENV 3 CH53489, DENV 4 341750) propagated in Vero cells. For the second experiment, DENV 4 strain 341750 was substituted with strain TVP360 because TVP 360 was originally used for the PIV production seed based on its ability to reach higher titers in the Vero cell substrate. Virus in the culture supernatant was concentrated by tangential flow ultrafiltration using a 100-kDa screen channel membrane (Filtron, Inc.), purified by sucrose gradient ultracentrifugation, and inactivated with formalin as originally described (Putnak et al., 1996). Monkeys were inoculated intramuscularly by needle and syringe in the upper arm (deltoid) with 4 µg (1 µg/serotype) of the TPIV formulation adjuvanted with 0.01% aluminum hydroxide (alum).

Preparation and administration of plasmid DNA vaccine

TDNA vaccines were prepared as previously described (Kochel et al., 1997). The vaccine constructs contained the DENV pre membrane (M) and full-length envelope (E) genes cloned into plasmid vector pVR1012 (Vical, San Diego, CA). The DENV 1 vaccine construct contained sequences from the Western Pacific 74 (West Pac 74) strain, whereas the plasmid constructs for DENVs 2, 3, and 4 contained sequences from near wild-type Philippine strains. The DENV 2 construct was modified by replacing the DENV transmembrane and cytoplasmic sequences with those of the mouse lysosome-associated membrane protein (LAMP) at the carboxy terminus of the E protein (D2MEL) (Raviprakash et al., 2001, 2003). Animals received 5 mg (1.25 mg/serotype) of TDNA vaccine per dose administered intramuscularly in the upper arm (deltoid), using the needle free Biojector system (Raviprakash et al., 2003).

Animal immunizations and virus challenge

For the first experiment, 16 healthy, flavivirus naïve, Indian origin rhesus macaques (*Macaca mulatta*) were randomized into four groups ($n=4$ animal/group). Group 1 was primed with two doses of the TDNA vaccine on day –30 and day 0, group 2 was primed once with TPIV vaccine on day 0, and group 3 was primed once with the TLAV vaccine on day 0. Groups 1–3 were then boosted with TLAV vaccine on day 60. Group 4 served as a control group and received saline only on days 0 and 60. Six months after, the TLAV booster the animals were challenged with live near wild-type DENV 3 (CH53489) PDK 0.

For the second experiment, 32 healthy, flavivirus naïve rhesus macaques were divided into two groups of 16 animals each. Sixteen animals received the TPIV vaccine on day 0 and the TLAV vaccine on day 60. The remaining 16 animals served as a control group and received saline on days 0 and 60. Six months after, the last immunization paired groups of four vaccinated and four control animals were challenged with each of the four dengue viruses, with one group challenged with DENV 1, another DENV-2, another DENV-3, and the final group receiving DENV-4. DENV challenges were administered by subcutaneous injection in the upper arm using 5 log₁₀ pfu of DENV 1 (West Pac 74), DENV 2 (S16803), DEN 3 (CH53489), or DEN 4 (341750), derived from wild-type virus isolates passaged only 3–4 times in Vero cells to make challenge virus stocks.

Viremia assay

The presence of virus in serum samples collected daily for 10 consecutive days after virus challenge was detected by incubation on Vero cell monolayers followed by immunofluorescent antibody (IFA) assay. Briefly, Vero 81 cells (ATCC) were propagated in 25 cm² flasks (T25) with EMEM, non-essential amino acids (BioWhittaker), 10% heat-inactivated FBS, and penicillin/streptomycin. Duplicate flasks were inoculated with 0.3 ml of a 1:2 dilution of each post-challenge serum sample and incubated at 37 °C for 14 days, with a complete media change on day 7. Cells from each flask were harvested by scraping, washed with PBS, and spotted in duplicate onto immunofluorescence slides. DENV-infected cells were detected by staining with anti-DENV serotype-specific monoclonal antibodies as well as HMAF followed by FITC-conjugated goat anti-mouse Ig. The cell fluorescence was scored as positive (+) or negative (−) compared to uninfected control cells.

Animal use statement

The research protocol using animals in this study was reviewed and approved by the Naval Medical Research Center's Animal Care and Use Committee according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals, National Research Council, NIH Publication no. 92-3415.

Acknowledgments

We thank the personnel of the Division of Veterinary Medicine of Walter Reed Army Institute of Research (WRAIR) for animal husbandry and technical assistance with the animal experiments. We gratefully acknowledge Michelle Ward and Obahi Omojuwanfo for technical assistance and Dr. Kenneth Eckels, WRAIR Pilot Bioproduction Facility, for providing the TLAV vaccine and challenge viruses.

This research was supported by Naval Medical Research Center work unit # 6000.RAD1.SA0312 and the U.S. Army Medical Research and Materiel Command.

The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. I am an employee of the U.S. Government. This work was prepared as part of my official duties. Title 17 U.S.C. article 105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. article 101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

References

- Barnett, S.W., Rajasekar, S., Legg, H., Doe, B., Fuller, D.H., Hayens, J.R., Walker, C.M., Steimer, K.S., 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp 120 protein subunit. *Vaccine* 15 (8), 869–873.
- Bancroft, W.H., Top Jr., F.H., Eckels, K.H., Anderson Jr., J.H., McCown, J.M., Russell, P.K., 1981. Dengue-2 vaccine: virological, immunological, and clinical responses of six yellow fever-immune recipients. *Infect. Immun.* 31 (2), 698–703.
- Bhamarapravati, N., Yoksan, S., 1997. Live-attenuated tetravalent dengue vaccine. In: Gubler, D.J., Kuno, G. (Eds.), *Dengue and Dengue Hemorrhagic fever*. CAB International, Cambridge, United Kingdom, pp. 367–377.
- Durbin, A.P., McArthur, J.H., Marron, J.A., Blaney Jr., J.E., Thumar, B., Wanionek, K., Murphy, B.R., Whitehead, S.S., 2006a. The live-attenuated dengue serotype 1 vaccine rDEN1Delta30 is safe and highly immunogenic in healthy adult volunteers. *Hum. Vaccine* 2 (4), 167–173 Jul-Aug.
- Durbin, A.P., McArthur, J.H., Marron, J.A., Blaney Jr., J.E., Thumar, B., Wanionek, K., Murphy, B.R., Whitehead, S.S., 2006b. rDEN2/4Delta30(ME), a live attenuated chimeric dengue serotype 2 vaccine is safe and highly immunogenic in healthy adult volunteers. *Hum. Vaccine* 2 (6), 255–260 Nov-Dec.
- Eckels, K.H., Dubois, D.R., Putnak, R., Vaughn, D.W., Innis, B.L., Henchal, E.A., Hoke Jr., C.H., 2003. Modification of dengue virus strain by passage in primary dog kidney cells: preparation of candidate vaccines and immunization of monkeys. *Am. J. Trop. Med. Hyg.* 69 (6 Suppl.), 12–16 Dec Erratum in: *Am. J. Trop. Med. Hyg.* 2004, Mar; 70(3):336.
- Guirakhoo, F., Kitchener, S., Morrison, D., Forrat, R., McCarthy, K., Nichols, R., Yoksan, S., Duan, X., Ermak, T.H., Kanasa-Thanan, N., Bedford, P., Lang, J., Quentin-Millet, M.J., Monath, T.P., 2006. Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: phase 1 clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. *Hum. Vaccine* 2 (2), 60–67 Mar-Apr.
- Guy, B., Barban, V., Mantel, N., Aguirre, M., Gulia, S., Pontvianne, J., Jourdiar, T.M., Ramirez, L., Gregoire, V., Charnay, C., Burdier, N., Dumas, R., Lang, J., 2009. Evaluation of interferences between dengue vaccine serotypes in a monkey model. *Am. J. Trop. Med. Hyg.* 80 (2), 302–311 Feb.
- Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* 239 (4839), 476–481 Jan 29.
- Halstead, S.B., Marchette, N.J., 2003. Biologic properties of dengue viruses following serial passage in primary dog kidney cells: studies at the University of Hawaii. *Am. J. Trop. Med. Hyg.* 69 (6 Suppl.), 5–11 Dec.
- Huang, C.Y., Butrapet, S., Tsuchiya, K.R., Bhamarapravati, N., Gubler, D.J., Kinney, R.M., 2003. Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development. *J. Virol.* 77 (21), 11436–11447 Nov.
- Kanasa-Thanan, N., Sun, W., Ludwig, G.V., Rossi, C., Putnak, J.R., Mangiafico, J.A., Innis, B.L., Edelman, R., 2003. Atypical antibody responses in dengue vaccine recipients. *Am. J. Trop. Med. Hyg.* 69 (6 Suppl.), 32–38 Dec Erratum in: *Am. J. Trop. Med. Hyg.* 2004, Mar; 70(3):336.
- Kaufman, B.M., Summers, P.L., Dubois, D.R., Eckels, K.H., 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* 36 (2), 427–434 Mar.
- Kitchener, S., Nissen, M., Nasveld, P., Forrat, R., Yoksan, S., Lang, J., Saluzzo, J.F., 2006. Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. *Vaccine* 24 (9), 1238–1241 Feb; 27 Epub. 2005, Sep 23.
- 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 Apr.
- McArthur, J.H., Durbin, A.P., Marron, J.A., Wanionek, K.A., Thumar, B., Pierro, D.J., Schmidt, A.C., Blaney Jr., J.E., Murphy, B.R., Whitehead, S.S., 2008. Phase 1 clinical evaluation of rDEN4Delta30-200, 201, a live attenuated dengue 4 vaccine candidate designed for decreased hepatotoxicity. *Am. J. Trop. Med. Hyg.* 79 (5), 678–684 Nov.
- Monath, T.P., 1986. Pathology of the flaviviruses. In: Schlesinger, S., Schlesinger, M.J. (Eds.), *The Togaviridae and Flaviviridae*. Plenum, New York, pp. 375–440.
- Putnak, R., Barvir, D., Dubois, D., Dandrea, V., Hoke, C., Sadoff, J., Eckels, K., 1996. Development of a purified, inactivated dengue 2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. *J. Infect. Dis.* 174, 1176–1184.
- Putnak, J.R., Collier, B.A., Voss, G., Vaughn, D.W., Clements, D., Peters, I., Bignami, G., Houg, H.S., Chen, R.C., Barvir, D.A., Seriwatana, J., Cayphas, S., Garcon, N., Gheysen, D., Kanasa-Thanan, N., McDonell, M., Humphreys, T., Eckels, K.H., Prieels, J.P., Innis, B.L., 2005. An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. *Vaccine* 23 (35), 4442–4452 Aug 15.
- 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 Nov 10.
- 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 Oct 25.
- 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.
- Sabchareon, A., Lang, J., Chantavanich, P., Yoksan, S., Forrat, R., Attanath, P., Sirivichayakul, C., Pengsaa, K., Pojarojan-anant, C., Choekjindachai, W., Jagsudee, A., Saluzzo, J., Bhamarapravati, N., 2002. Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio and multiple doses. *Am. J. Trop. Med. Hyg.* 66 (3), 264–272.
- Scott, R.M., Eckels, K.H., Bancroft, W.H., Summer, P.L., McCown, J.M., Anderson, J.H., Russell, P.K., 1983. Dengue 2 vaccine: dose response in volunteers in relation to yellow fever immune status. *J. Infect. Dis.* 148 (6), 1055–1060.
- Sedegah, M., Jones, T.R., Kaur, M., Hedstrom, R., Hobart, P., Tine, J.A., Hoffman, S.L., 1998. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc. Natl. Acad. Sci.* 95, 7648–7653.
- Simmons, M., Murphy, G.S., Kochel, T., Raviprakash, K., Hayes, C.G., 2001. Characterization of antibody responses to combinations of a dengue-2DNA and dengue-2 recombinant subunit vaccine. *Am. J. Trop. Med. Hyg.* 65 (5), 420–426.
- Simmons, M., Porter, K.R., Hayes, C.G., Vaughn, D.W., Putnak, R., 2006. Characterization of antibody responses to combinations of a dengue virus type 2 DNA vaccine and two dengue virus type 2 protein vaccines in rhesus macaques. *J. Virol.* 80 (19), 9577–9585.

- Sun, W., Edelman, R., Kanesa-Thasan, N., Eckels, K.H., Putnak, J.R., King, A.D., Houn, H.S., Tang, D., Scherer, J.M., Hoke Jr., C.H., Innis, B.L., 2003. Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. *Am. J. Trop. Med. Hyg.* 69 (6 Suppl.), 24–31 Dec.
- Sun, W., Nisalak, A., Gettayacamin, M., Eckels, K.H., Putnak, J.R., Vaughn, D.W., Innis, B.L., Thomas, S.J., Endy, T.P., 2006. Protection of rhesus monkeys against dengue virus challenge after tetravalent live attenuated dengue virus vaccination. *J. Infect. Dis.* 193 (12), 1658–1665 Jun 15.
- Sun, W., Cunningham, D., Wasserman, S.S., Perry, J., Putnak, J.R., Eckels, K.H., Vaughn, D. W., Thomas, S.J., Kanesa-Thasan, N., Innis, B.L., Edelman, R., 2009. Phase 2 clinical trial of three formulations of tetravalent live-attenuated dengue vaccine in flavivirus-naïve adults. *Hum. Vaccine* 5 (1), 33–40 Jan-Feb.