

A dengue DNA vaccine formulated with Vaxfectin® is well tolerated, and elicits strong neutralizing antibody responses to all four dengue serotypes in New Zealand white rabbits

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A tetravalent DNA vaccine formulated with Vaxfectin adjuvant was shown to elicit high levels of neutralizing antibody against all four dengue virus serotypes (Porter et al., 2011¹⁶), warranting further testing in humans. In preparation for a phase 1 clinical testing, the vaccine and the adjuvant were manufactured under current good manufacturing practice guidelines. The formulated vaccine and the adjuvant were tested for safety and/or immunogenicity in New Zealand white rabbits using a repeat dose toxicology study. The formulated vaccine and the adjuvant were found to be well tolerated by the animals. Animals injected with formulated vaccine produced strong neutralizing antibody response to all four dengue serotypes.

Dengue viruses belong to the family *Flaviviridae*. Four antigenically distinct serotypes of dengue virus have similar clinical presentation, epidemiology, and distribution, especially in the tropical and subtropical regions of the world, where nearly 2.5 billion people are at risk of infection.¹ Infection with any of the four dengue virus serotypes can cause diseases ranging from mild febrile illness and classic dengue fever to the severe and potentially fatal forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).² Natural infection with any of the dengue virus serotypes provides only long-term homotypic immunity, and available epidemiologic data suggest an increased risk for DHF/DSS during secondary infections with a heterologous serotype.^{3,4} Global expansion of dengue virus infections in recent decades has made the development of vaccines for dengue viruses a public health priority. Traditional vaccine approaches such as live attenuated viruses,^{5,6} inactivated viruses,⁷ and subunit vaccines,⁸ as well as novel approaches such as cloned, engineered viruses⁹ and chimeric viruses using yellow fever virus backbone¹⁰ are being pursued. Several have advanced to clinical evaluation; however, a licensed vaccine is not yet available.

To avoid the potential for increased risk of DHF/DSS due to postulated immune enhancement,¹¹ a dengue virus vaccine should elicit immunity simultaneously to all four serotypes. We have developed plasmid DNA vaccines directed against each of the four dengue serotypes that express respective pre-membrane and envelope antigens. These have been tested in small animal

and non-human primate models.^{12–14} A nonadjuvanted, monovalent dengue-1 DNA vaccine was recently tested in a phase 1 proof-of-concept clinical trial in which modest antibody responses were demonstrated in a subset of vaccinated individuals.¹⁵ In order to enhance antibody responses, we recently tested in rhesus macaques a tetravalent dengue DNA vaccine (a mixture of 4 plasmids encoding the 4 dengue antigens) formulated with Vaxfectin® adjuvant. Significantly higher, and longer lasting, neutralizing antibodies to all four serotypes were demonstrated compared with vaccine without the adjuvant,¹⁶ warranting further investigation in a phase 1 clinical trial. Here, we report the safety and immunogenicity results of a Vaxfectin®-adjuvanted tetravalent dengue DNA vaccine (TV DV) in New Zealand white rabbits. This study was conducted according to Good Laboratory Practices (GLP) in support of an Investigational New Drug application (IND).

Plasmids expressing pre-membrane and envelope proteins of dengue virus type-1, -2 and -3 have been described previously.^{12–14} Minor changes were introduced in the sequences just upstream of the initiating methionine codon in order to remove certain redundant sequences. A dengue-4 vaccine construct similar to the other plasmids was prepared. All four DNA plasmid constructs were manufactured by Vical Inc. according to current Good Manufacturing Practice (cGMP) guidelines. The plasmids were mixed (1:1:1:1, wt/wt) to produce TV DV. Vaxfectin®,¹⁷ a cationic lipid-based adjuvant also manufactured by Vical according to cGMP guidelines, was combined with TV DV to produce

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an adjuvanted vaccine containing both DNA and lipid at final concentrations of 1 mg/mL (TVDV^{Vax}).

Three groups of 16 New Zealand white rabbits (8 males and 8 females) were vaccinated with phosphate buffered saline (PBS control, group 1), TVDV^{Vax} at 2 mg (group 2, 0.5 mg DNA of each of the 4 vaccine constructs) or Vaxfectin[®] at 4 mg (group 3). All treatments were delivered as bilateral 1 mL injections to the *vastus lateralis*. The TVDV^{Vax} dose was selected based on the proposed clinical protocol. The Vaxfectin[®] alone group was included to assess the potential contribution of Vaxfectin[®] adjuvant to any responses observed in the TVDV^{Vax} group; as the adjuvant was supplied at a 2 mg/mL concentration, delivering bilateral 1 mL injections yielded a 4 mg total body dose. Animals were vaccinated on Days 1, 30, 60 and 90. Euthanasia time-points were scheduled for Day 92 (acute toxicity, 48 h following final dose) and 120 (recovery toxicity, 30 d following final dose). Parameters evaluated included clinical observations, dermal irritation, body weight and temperature, food consumption, clinical pathology, ophthalmology, gross necropsy findings, absolute and relative organ weights, and histopathology findings. Serum samples were also prepared from blood collected on Day 60 (post 2nd dose) and Day 120 (post 4th dose) and used for measurement of neutralizing antibody titers by plaque reduction neutralization test (PRNT).¹⁸

One group 3 female was found dead on Day 62. This animal had mistakenly received 6 mg of Vaxfectin[®] instead of 4 mg on Day 30. Because no unusual clinical, gross or microscopic observations were seen, the death could not be attributed to test article administration and the cause of death was undetermined. All other animals survived until scheduled euthanasia. Cage side observations and dermal irritation scoring revealed the presence of varying degrees of erythema and edema in all groups, including PBS controls. Erythema and edema were increased in group 2 and 3 males when compared with controls, but were minor in extent (scoring very slight to slight) and duration (resolving in 1–5 d), and did not appear different between the test article groups 2 and 3. Body weights and body weight changes during the study were mostly unremarkable, and there were no statistically significant test article-related changes. Ophthalmic examinations were normal across groups.

There were no test article-related changes in organ weights at necropsy. Microscopic examination did reveal pyogranulomatous and/or granulomatous inflammatory lesions at both injection site skin and muscle in both TVDV^{Vax}- and Vaxfectin[®]-treated rabbits. The incidence of muscle lesions in male and female animals and skin lesions in females was higher with TVDV^{Vax} than with Vaxfectin[®] on Day 92 (acute toxicology time point). Recovery was apparent by Day 120.

Clinical chemistry/pathology panels revealed several test article-related changes that were likely secondary to the injection site inflammation, generally occurring on Day 3 and 92 (48 h post-dosing) but resolving by later time-points. These included elevated levels of total white blood cells, segmented neutrophils, and lymphocytes in group 2 males, and elevated segmented neutrophils in groups 2 and 3 females (Table 1). On study day 3, somewhat decreased levels of hemoglobin and hematocrit were

noted in group 2 males and females. Liver function tests indicated elevated levels of aspartate aminotransferase (AST) in group 2 animals on study day 92 (Table 2). These responses were transient and had resolved by study day 120 (not shown). Forty-eight hours after the first administration of test articles (study day 3), animals in both groups 2 and 3 had decreased prothrombin times and elevated activated partial thromboplastin times (Table 3). Both test article treated groups also showed elevated fibrinogen levels on days 3 and 92. C-reactive protein (CRP), which was included as an inflammatory marker endpoint, was also transiently increased in both test article groups at Days 3 and 92 (Table 2). This marker (CRP) rises but quickly resolves, providing a sensitive measure of inflammatory response. Transient increases in CRP in this study offer additional support for concluding that the clinical pathology changes observed were likely secondary to injection site reactogenicity/inflammation related to intramuscular injection of TVDV^{Vax} or Vaxfectin[®].

Dengue virus neutralizing antibodies were determined for Days 60 and 120 sera of all TVDV^{Vax} vaccinated animals and two each of PBS and Vaxfectin[®] vaccinated animals. No neutralizing antibodies against any of the dengue serotypes were detected in animals vaccinated with PBS or Vaxfectin[®] alone (not shown). Geometric mean 50% neutralization titers for each of the 4 dengue serotypes are shown in Figure 1. After two doses of TVDV^{Vax}, all animals had seroconverted to all 4 serotypes except one male and one female animal in which antibody to dengue-4 could not be detected. The 50% neutralization titers ranged between 128–716 (dengue-1), 156–672 (dengue-2), 128–716 (dengue-3) and 38–140 (dengue-4). By Day 120, however, 100% of the animals had significant levels of neutralizing antibody to all 4 serotypes. The range of antibody titers had increased to 724–1349 (dengue-1), 1287–2454 (dengue-2), 848–1632 (dengue-3) and 194–290 (dengue-4). From Day 60 (n = 16) to Day 120 (n = 8), the geometric mean titers had increased from 261 to 1032 (dengue-1), 253 to 1729 (dengue-2), 222 to 1107 (dengue-3) and 41 to 241 (dengue-4).

These results demonstrate that repeated intramuscular injections of 2 mg TVDV^{Vax} or 4 mg Vaxfectin[®] were well tolerated by rabbits. The observations and findings made during this toxicology study were representative of the type of changes expected following the intramuscular injection of an adjuvanted vaccine such as TVDV^{Vax}, and are characteristic of the successful induction of an acquired immune response. Similar qualitative changes were seen following delivery of Vaxfectin[®] (at double the dose present in TVDV^{Vax}) but with some indications of a lower degree of severity (e.g., as with the Day 92 inflammatory lesions). The Vaxfectin[®]-related findings are in agreement with a previous report¹⁹ and once again are not unexpected for an adjuvant whose proposed mechanism of action is the induction/modulation of immune responses.¹⁷ In addition, all animals elicited strong neutralizing antibodies to all 4 dengue serotypes. The antibody responses were much higher than previously reported for monovalent vaccine constructs in mice and monkeys,^{12–14,18} or for Vaxfectin[®]-adjuvanted TVDV in monkeys.¹⁶ This could be due to the differences in animal species, or different doses used in different animal species. It will be interesting to see how

Table 1. Key hematology indicators.

	Study Day 1						Study Day 3					
	Grp 1		Grp 2		Grp 3		Grp 1		Grp 2		Grp 3	
	M	F	M	F	M	F	M	F	M	F	M	F
WBC (1E+3/uL)	6.88 (1.19)	6.25 (1.2)	7.98 (1.75)	7.18 (1.7)	7.07 (0.56)	6.24 (0.98)	7.93 (1.51)	7.53 (1.22)	11.06 (1.81)	8.58 (2.5)	7.63 (0.82)	6.53 (1.35)
RBC (1E+6/uL)	6.35 (0.43)	6.25 (0.35)	6.32 (0.27)	6.1 (0.37)	6.66 (0.43)	6.07 (0.44)	5.78 (0.39)	5.87 (0.3)	5.3 (0.39)	5.26 (0.28)	6.06 (0.43)	5.79 (0.45)
HGB (g/dL)	13.35 (0.42)	13.36 (0.62)	13.58 (0.57)	13.21 (0.49)	13.89 (0.86)	12.91 (0.75)	12.3 (0.45)	12.43 (0.5)	11.21 (0.62)	11.32 (0.43)	12.6 (0.63)	12.1 (0.88)
HCT (%)	41.61 (1.15)	41.28 (1.96)	42.11 (1.62)	40.69 (1.71)	42.84 (2.5)	39.89 (2.42)	37.99 (1.14)	38.55 (1.55)	34.81 (2.0)	34.86 (1.38)	38.93 (1.9)	37.57 (2.85)
Platelets (1E+3/uL)	405.9 (82)	222.5 (115)	343.1 (103)	378.4 (112)	338.7 (143)	404.9 (107)	412.6 (85.5)	358.3 (64.8)	278.4 (164)	148.3 (75.7)	318.3 (13.3)	332 (201)
SEGS (1E+3/uL)	1.17 (0.59)	0.92 (0.67)	0.89 (0.22)	1.57 (0.87)	0.79 (0.19)	1.20 (0.48)	1.37 (0.50)	1.6 (0.48)	4.53 (1.36)	1.95 (0.69)	1.29 (0.28)	1.82 (0.80)
LYMPHS (1E+3/uL)	5.28 (1.21)	4.95 (0.88)	6.65 (1.56)	5.01 (1.02)	5.97 (0.38)	4.67 (0.97)	5.69 (1.33)	5.28 (1.38)	5.8 (1.89)	6.1 (2.35)	5.76 (0.44)	4.08 (1.15)

	Study Day 90						Study Day 92					
	M	F	M	F	M	F	M	F	M	F	M	F
WBC (1E+3/uL)	8.13 (0.88)	7.26 (0.82)	9.54 (2.16)	7.68 (2.58)	7.61 (1.53)	6.61 (1.21)	8 (0.77)	10.13 (1.69)	14.7 (0.95)	11.4 (2.84)	9.48 (1.8)	10.13 (1.72)
RBC (1E+6/uL)	6.37 (0.34)	6.02 (0.51)	6.43 (0.40)	6.02 (0.40)	6.74 (0.59)	6.12 (0.35)	5.98 (0.45)	6.14 (0.41)	5.91 (0.52)	5.77 (0.56)	5.94 (0.52)	5.89 (0.31)
HGB (g/dL)	13.93 (0.31)	12.81 (0.8)	13.94 (0.86)	12.76 (.53)	14.24 (1.02)	12.86 (0.65)	13.23 (0.6)	12.88 (0.73)	12.43 (1.06)	12.03 (.87)	12.53 (0.64)	12.03 (0.41)
HCT (%)	41.33 (0.91)	37.88 (2.46)	41.74 (2.36)	38.59 (1.57)	42.91 (2.96)	38.9 (1.9)	38.45 (1.88)	38.23 (2.37)	37.28 (2.87)	36.2 (2.23)	38.28 (2.08)	36.68 (1.42)
Platelets (1E+3/uL)	309 (79.8)	233.9 (48.2)	308.3 (71.5)	254.8 (77.2)	274.5 (54.1)	227.7 (79.1)	274.3 (73.8)	231.5 (86.7)	196.3 (42.9)	160.3 (91.3)	323.3 (102.1)	251.8 (11.8)
SEGS (1E+3/uL)	1.12 (0.38)	1.16 (0.43)	1.10 (0.50)	1.59 (0.72)	1.15 (0.8)	1.25 (0.47)	0.88 (0.25)	1.31 (0.66)	4.5 (1.39)	2.88 (1.15)	1.9 (0.74)	3.12 (0.91)
LYMPHS (1E+3/uL)	6.53 (0.64)	5.56 (0.89)	7.59 (1.83)	5.67 (2.14)	5.76 (1.38)	5.08 (0.89)	6.55 (0.72)	8.06 (1.29)	9.46 (0.59)	7.76 (1.70)	6.67 (1.64)	6.12 (0.89)

Groups 1, 2 and 3 are treated with PBS, TVDV and Vaxfectin respectively. M and F refer to male and female animals. Shown are group averages with standard deviation in parentheses for white and red blood cells (WBC, RBC), hemoglobin (HGB), hematocrit (HCT), platelets, segmented neutrophils (SEGS) and lymphocytes (LYMPHS). Group 2 and 3 values that are significantly higher or lower compared to group 1 ($p < 0.05$) are shown in the shaded or dotted boxes respectively.

this adjuvanted DNA vaccine will perform in humans. A phase I clinical trial of this vaccine was initiated in early 2012.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 2. Clinical chemistry

	Study Day 1						Study Day 3					
	Grp 1		Grp 2		Grp 3		Grp 1		Grp 2		Grp 3	
	M	F	M	F	M	F	M	F	M	F	M	F
AST (u/L)	19 (13.7)	11.1 (2.1)	19.5 (10.6)	22.1 (11.5)	21.1 (9.9)	16.9 (10.2)	16.4 (5.1)	14 (5.5)	18.8 (6.9)	20.6 (6.7)	18.1 (9.8)	22.8 (31.8)
ALT (u/L)	47.9 (9.1)	31.4 (19.1)	38 (16.8)	49.6 (20.7)	37.6 (13.3)	47.3 (15.9)	41.3 (9.9)	39.3 (11.1)	38.0 (13.2)	41.4 (7.6)	34.8 (14.3)	48.5 (25.1)
CRP (ug/mL)	0.00	3.52 (3.69)	0.00	8.41 (14.16)	0.63 (0.68)	3.48 (3.51)	2.39 (6.47)	9.34 (7.87)	41.37 (4.82)	33.28 (5.42)	32.06 (6.45)	26.66 (6.14)
	Study Day 90						Study Day 92					
	Grp 1		Grp 2		Grp 3		Grp 1		Grp 2		Grp 3	
	M	F	M	F	M	F	M	F	M	F	M	F
AST (u/L)	13.6 (5.8)	9.9 (3.7)	21.1 (24.6)	16.6 (10.3)	13.1 (5.7)	11.7 (5.6)	12.0 (2.9)	13.0 (3.5)	28.8 (7.3)	26.5 (6.5)	17.3 (3.8)	12.8 (1.5)
ALT (u/L)	51.1 (9.1)	40.4 (18.6)	60.0 (49.9)	50.6 (15.6)	40.1 (13.0)	49.0 (16.4)	55.8 (6.7)	38.8 (16.5)	59.8 (34.5)	51.3 (6.2)	49.0 (14.2)	60.5 (13.7)
CRP (ug/mL)	0.37 (0.77)	7.05 (6.87)	1.0 (1.08)	5.16 (5.26)	11.93 (24.78)	3.55 (4.61)	0.0 (0.0)	19.6 (29.58)	287.3 (107.4)	239.1 (133.5)	147.8 (45.8)	120.7 (17.8)

Groups 1, 2 and 3 are treated with PBS, TVDV and Vaxfectin respectively. M and F refer to male and female animals. Shown are group averages with standard deviation in parentheses for alanine and aspartate amino- transferases (ALT and AST), and C-reactive protein (CRP). Group 2 and 3 values that are significantly higher or lower compared to group 1 ($p < 0.05$) are shown in shaded or dotted boxes respectively.

Table 3. Coagulation tests

	Study Day 1						Study Day 3					
	Grp 1		Grp 2		Grp 3		Grp 1		Grp 2		Grp 3	
	M	F	M	F	M	F	M	F	M	F	M	F
PT (sec)	6.49 (0.15)	6.5 (0.61)	6.9 (1.68)	6.3 (0.12)	8.26 (5.51)	6.98 (1.81)	6.14 (0.14)	6.26 (0.21)	5.79 (0.1)	5.81 (0.14)	5.94 (0.15)	5.93 (0.12)
APT (sec)	49.65 (9.3)	52.53 (11.67)	49.93 (12.03)	46.6 (22)	49.43 (11.3)	54.46 (14.74)	54.64 (4.4)	50 (5.2)	93.8 (13.1)	82.4 (11.2)	71.6 (13)	57 (16.3)
FIB (mg/dL)	282.4 (29.3)	227.5 (70.3)	233.9 (113.8)	207.1 (94.3)	250.9 (96.1)	233.9 (79.6)	255 (55)	221.3 (28.1)	943.1 (162)	600.6 (99.3)	522.1 (105)	420.6 (103.7)
	Study Day 90						Study Day 92					
	Grp 1		Grp 2		Grp 3		Grp 1		Grp 2		Grp 3	
	M	F	M	F	M	F	M	F	M	F	M	F
PT (sec)	6.54 (0.84)	7.58 (3.1)	6.56 (0.34)	6.66 (0.48)	6.4 (0.19)	7.9 (4.1)	6.25 (0.1)	7.08 (1.75)	6.05 (0.06)	6.05 (0.17)	6.13 (0.17)	6.18 (0.22)
APT (sec)	53.11 (5.8)	51.65 (10.78)	42.75 (9.13)	53.03 (9.53)	51.98 (4.72)	53.06 (6.88)	55.1 (7.9)	49.9 (9.18)	78.0 (8.98)	66.43 (11.0)	63.4 (8.02)	62.15 (6.21)
FIB (mg/dL)	201.3 (113.1)	109.4 (58)	130.6 (67)	126.3 (46.5)	255.4 (66.2)	149.7 (41.7)	217.8 (54.4)	177.3 (126.7)	689.8 (89.9)	558.3 (111.4)	668.8 (51.9)	389.3 (32.8)

Groups 1, 2 and 3 are treated with PBS, TVDV and Vaxfectin respectively. M and F refer to male and female animals. Shown are group averages with standard deviation in parentheses for prothrombin time (PT), activated prothrombin time (APT) and fibrinogen protein. Group 2 and 3 values that are significantly higher or lower compared to group 1 ($p < 0.05$) are shown in shaded or dotted boxes respectively.

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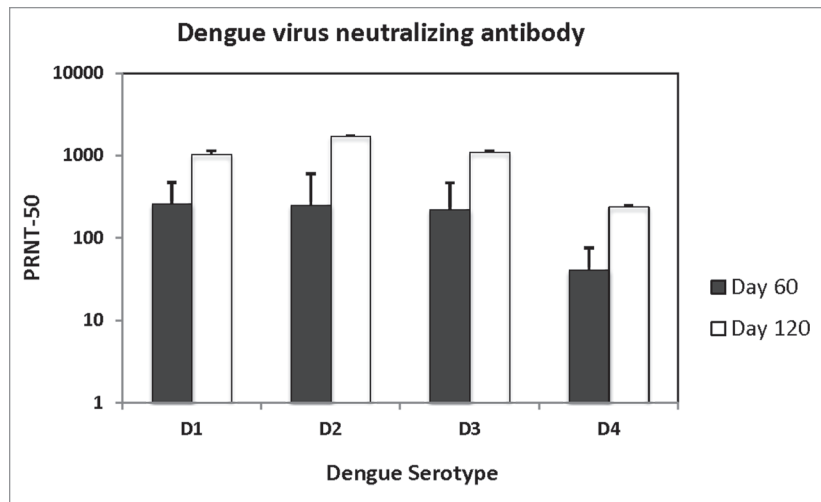


Figure 1. Virus neutralizing antibody in rabbits vaccinated with TVDV^{Vax}. Geometric mean fifty percent virus neutralization titers (PRNT-50) for each of the 4 dengue serotypes (D1–D4) using sera after 2 doses of vaccine (Day 60) and 4 doses of vaccine (Day 120) are shown.

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