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Immunogenicity and protective efficacy of a recombinant fusion protein containing the domain III of the dengue 1 envelope protein in non-human primates

Lidice Bernardo[a], Alienys Izquierdo[a], Mayling Alvarez[a], Delfina Rosario[a], Irina Prado[a], Carlos López[b], Rafael Martínez[b], Jorge Castro[b], Emidalys Santana[a], Lisset Hermida[b], Gerardo Guillen[b], María G. Guzmán[a,*]

[a] *Department of Virology, PAHO/WHO Collaborating Center for the Study of Dengue and its Vector, "Pedro Kourí" Tropical Medicine Institute (IPK), Autopista Novia del Mediodía, km 6 1/2, P.O. Box 601, Marianao 13, Havana, Cuba*
[b] *Center for Genetic Engineering and Biotechnology, Apdo 6162, Havana 10 600, Cuba*

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ABSTRACT

Recombinant fusion proteins containing the aa 286–426 of the dengue envelope protein fused to P64k protein from *Neisseria meningitidis* have been previously reported. Particularly, the immunogenicity and protective capacity of the dengue 2 recombinant protein was demonstrated in *Macaca fascicularis* monkeys. Here we evaluate the recombinant fusion protein containing the domain III of the dengue 1 envelope protein (PD10) in non-human primates (*M. fascicularis* and rhesus monkeys) and compare the effect of aluminum hydroxide and Freund adjuvant on the immunity induced. The PD10 protein emulsified in Freund adjuvant was highly immunogenic in *M. fascicularis* and rhesus monkeys. Following dengue 1 virus challenge, animals immunized with PD10 in Freund adjuvant were protected from viremia. However, monkeys receiving PD10 in aluminum hydroxide developed a poor antibody response and were not protected from viral challenge. These preliminary experiments are encouraging. Other formulations or vaccine schedules are being studied in an attempt to find regimens that enhance immunological protection.

1. Introduction

Dengue virus (DENV) infections have become a major worldwide public health problem throughout the tropical and subtropical areas of the world. Vector control, which has proven difficult and costly to sustain over time, is the only available control measure (Gubler and Clark, 1994; Guzmán and Kourí, 2002). Decades of effort have not produced a successful DENV vaccine so far. Based on the lack of cross-protective immunity among the four DENV serotypes (Rothman, 2004) and the higher risk for severe DENV disease during secondary heterologous infection (Alvarez et al., 2006; Guzmán et al., 2006; Halstead and O'Rourke, 1977), it is widely believed that a DENV vaccine must be tetravalent, capable of protecting against infections by the four serotypes (Halstead and Deen, 2002; Hombach et al., 2005). This consideration, together with the lack of a suitable animal model that reproduces disease symptoms, has made the development of a DENV vaccine a challenging task (Hombach et al., 2005). The *Macaca mulatta* (rhesus) monkeys are considered the most appropriate preclinical animal model for assessing dengue virus infections. The level of viremia tends to be lower than in humans and there is no clinically apparent disease, but the serological response is similar to the human immune response (Guirakhoo et al., 2000; Halstead and Palumbo, 1973; Halstead et al., 1973; Sariol et al., 2007). Because of the limited supply of rhesus monkeys, other non-human primate species such us *M. fascicularis* have also been successfully employed for dengue vaccine evaluations (Hermida et al., 2006; Koraka et al., 2007; Velzing et al., 1999). Nevertheless, the advantages/disadvantages of both monkey species for vaccine studies deserve careful attention.

Although early efforts to develop a DENV vaccine have focused on conventional approaches (Saluzzo, 2003), considerable research work has also been directed toward the development of a subunit DENV vaccine. The recombinant subunit proteins made by the expression of genes encoding the DENV envelope (E) protein, with or without the pre-membrane (prM) protein, are immunogenic and confer some degree of protection against homologous viral challenge in animal models (Guzmán et al., 2003; Men et al., 1991; Muné et al., 2003; Putnak et al., 1991, 2005; Simmons et al., 2006; Srivastava et al., 1995). In recent years, the DENV E domain III has emerged as a promising subunit vaccine candidate (Hermida et al.,

* Corresponding author. Tel.: +53 7 2020450; fax: +53 7 2046051.
E-mail address: lupe@ipk.sld.cu (M.G. Guzmán).

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2004a,b, 2006; Khanam et al., 2006a,b; Mota et al., 2005; Simmons et al., 1998, 2001, 2006). DENV E domain III has been implicated in the interaction with cellular receptors in human and mosquito cells, even taking into account that specific cellular receptors for dengue viruses have not been identified. In addition, the recombinant domain III E proteins have induced antibodies that completely neutralized homologous dengue serotype up to 1:16 serum dilution (Chin et al., 2007).

We have previously demonstrated that the recombinant fusion proteins containing amino acids 286–426 of the DENV-1 and -2 E proteins (domain III) fused to the meningococcal P64k protein (Zulueta et al., 2003) are immunogenic and protective in mice (Hermida et al., 2004a,b). Additionally, the DENV-2 recombinant fusion protein induced functional antibodies and protective immune response in *M. fascicularis* monkeys (Hermida et al., 2006). Freund's adjuvant was used to induce the optimal immune response in the proof-of-concept analysis in non-human primates. However, other adjuvants must be evaluated before clinical trials. Aluminum is the most widely used adjuvant in both human and veterinary vaccines and generally stimulates a Th2 immune response (Lindblad, 2004).

The goals of this study were (1) to evaluate the immunogenicity and the protective capacity induced by the recombinant fusion protein containing the domain III of DENV-1 E protein fused to the P64k protein from *Neisseria meningitidis* in non-human primates, (2) to compare the immune response induced by the DENV-1 recombinant protein in *M. fascicularis* and rhesus monkeys and (3) to assess the immune response induced by the DENV-1 recombinant protein in Freund's and aluminum hydroxide adjuvant using the rhesus monkeys model.

2. Material and methods

2.1. Animals

Four adult *M. fascicularis* monkeys (between 2 and 4 kg of weight) and six adult rhesus monkeys (between 5 and 7 kg of weight) were used in this study. The monkeys were prescreened for dengue and P64k-specific antibodies. Only those animals that did not show any evidence of previous exposure to dengue or P64k were included in the study. Monkeys were maintained in accordance with the Cuban guidelines for the care and use of laboratory animals.

2.2. Recombinant proteins

The design, cloning, expression and initial evaluation of the DENV-1 recombinant fusion protein have been previously described (Zulueta et al., 2003). In brief, the DENV-1 E gene fragment from the Jamaica strain (isolated in 1977, CV11636, generously donated by the late Dr. Robert Shope), coding for aa 286–426, was cloned into an *Escherichia coli* vector containing the sequence of P64k protein. The DENV-1 recombinant protein (PD10) resulted from the fusion of the domain III E fragment to the C-terminus of the P64k protein (Zulueta et al., 2003). The P64k protein alone was used as negative control.

2.3. Cells and viruses

African green monkey kidney (Vero) cells and baby hamster kidney (BHK-21) cells were grown at 37 °C in 199 medium and Eagle's minimal essential medium (E-MEM), respectively. The media were supplemented with 10% heat-inactivated fetal bovine serum (HFBS). *Aedes albopictus* cell line (C6/36-HT) was grown at 33 °C in E-MEM supplemented with 10% HFBS, 1% non-essential amino acids and 1% glutamine solution (200 mM).

The standard strain DENV-1 Hawaii grown in suckling mouse brain and extracted by the sucrose–acetone method was used for enzyme-linked immunosorbent assay (ELISA). The plaque reduction neutralization test (PRNT) (Alvarez et al., 2005; Morens et al., 1985) was performed with supernatants from C6/36-HT cells, infected with the strain DENV-1 West Pacific (isolated in 1974, generously supplied by the WHO expert committee on biological standardization). For the challenge assay, a viral stock of DENV-1 Jamaica strain was prepared in Vero cells as previously described (Hermida et al., 2006) and titrated by the plaque method in BHK-21 cells (Alvarez et al., 2005; Morens et al., 1985).

2.4. Immunizations

Groups of two adult monkeys (*M. fascicularis* and rhesus, one male and one female per group) were immunized subcutaneously with 100 μg of either PD10 or P64k protein in Freund adjuvant. The recombinant proteins (100 μg/0.25 mL) were emulsified with 0.25 mL of complete Freund adjuvant for the first dose and incomplete Freud adjuvant for the following doses. In addition, two rhesus monkeys were immunized with PD10 in aluminum hydroxide. Aluminum hydroxide emulsion was prepared at 1.45 mg/mL and monkeys were inoculated with 0.5 mL of the mix. Four doses were administered to each group at days 0, 30, 90 and 150. Before each handling, the animals were intramuscularly anaesthetized with ketamine hydrochloride (5 mg/kg). Because of the limited supply of monkeys, use of aluminum hydroxide adjuvant and immunogenicity of P64k protein were not studied in *M. fascicularis* monkeys.

2.5. Virus challenge and viremia

At day 210 (2 months after the last immunization dose), all animals were subcutaneously inoculated in the upper arms with 6 log PFU of the DENV-1 Jamaica strain. To measure viremia, each monkey was bled prior to challenge and daily for 10 days. Viremia was detected by cell culture inoculation followed by an indirect immunofluorescence assay, immunocytochemical focus-forming (IFF) assay and reverse transcription-polymerase chain reaction (RT-PCR).

Viral isolation was performed by inoculating 100 μL of undiluted serum onto Vero cells grown in 24-well plates as previously described (Hermida et al., 2006; Rodriguez-Roche et al., 2000). In the sera that were positive for viral isolation, viremia was quantified by IFF using C6/36-HT cells. Undiluted or twofold diluted sera from 2.5 up to 10 were inoculated onto C6/36-HT cell suspension, previously seeded onto 6-well plates, and incubated for 4 h at 33 °C and 4% CO_2 for adsorption. Cells were overlaid with 3% medium viscosity carboxymethylcellulose (Sigma–Aldrich, Germany) prepared in E-MEM with 10% HFBS and 1% glutamine solution (200 mM) and incubated for 4 days at 33 °C and 4% CO_2. Cell monolayers were fixed for 2 h by the addition of 5 mL of 20% methanol and then washed with phosphate-buffered saline (PBS). Non-specific binding sites were blocked with PBS containing 2% skim milk for 1 h at room temperature. Cells were treated with an anti-DENV-1 hyperimmune mouse ascitic fluid for 1 h at room temperature. After three washes with PBS, the cells were incubated with anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Sigma, St. Louis, MO) for 1 h at room temperature. Plates were washed three times with PBS and antibody-bound foci of infection were developed by the addition of 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium and counted. Titers were expressed as focus-forming units (FFU) per milliliter.

Qualitative RT-PCR was carried out according to Lanciotti et al. (1992) with some modifications (Lanciotti et al., 1992; Rosario et al., 1998).

2.6. Antibody analysis

To evaluate the antibody response after vaccination, monkeys were bled at each of the immunization days and 15 days following an immunization day. In addition, the anamnestic antibody response after virus challenge was analyzed in the sera collected for viremia detection (days 1–10) and in four additional samples taken at days 15, 21, 30 and 60.

DENV-1-specific IgG antibodies in each individual serum sample were titrated by ELISA (Hermida et al., 2006). In brief, MAXISORP 96-well plates were coated with the monoclonal antibody 4G2 (ATCC), which recognizes the flavivirus E protein. Three washes with phosphate-buffered saline containing 0.05% Tween 20 (Merck, Germany) were completed after each step of the ELISA. Plates were blocked with 2% bovine serum albumin, and then incubated overnight at 4 °C with a saturating concentration of DENV antigen and the mock antigen in separate wells. Twofold diluted serum samples were incubated 1 h at 37 °C with either the DENV or the mock antigen. Anti-monkey IgG-peroxidase conjugate (Sigma) diluted 1/10,000 was incubated for 1 h at 37 °C. H_2O_2/*O*-phenylenediamine was added as substrate solution for 30 min. Optical densities (ODs) were measured at 492 nm. A dilution of serum was considered positive when the ratio [OD (DEN antigen)]/[OD (mock)] was two or higher.

Neutralizing antibodies were detected by PRNT in BHK-21 cells according to Morens et al. (1985) with some modifications (Alvarez et al., 2005; Morens et al., 1985). The serum dilution that resulted in a 50% reduction of plaque count ($PRNT_{50}$), as determined by probit analysis, was considered the neutralizing antibody titer.

3. Results

3.1. Antibody response after immunizations

The anti-DENV-1 antibodies induced after monkey immunization were analyzed by ELISA in the sera collected at the time of and 15 days after each dose (Fig. 1). The animals immunized with PD10 protein adjuvated in Freund (*M. fascicularis* and rhesus) showed high anti-DENV-1-specific IgG antibodies 15 days after the second dose. A booster effect was observed after the third and fourth doses and the antibodies declined over time. However, the rhesus monkeys immunized with PD10 protein in aluminum hydroxide showed low anti-DENV-1 IgG antibodies after immunization.

The neutralizing effect against DENV-1 was tested by PRNT in the sera collected from immunized animals (Table 1). All monkeys immunized with PD10 protein in Freund showed neutralizing antibodies 15 days after the third dose. The neutralizing antibody titers were boosted after the fourth dose and declined over time. By the day of challenge, only the monkey 1243 showed positive neutralizing antibody values. However, animals immunized with PD10 in aluminum hydroxide did not develop neutralizing antibodies after vaccination.

3.2. Protection of monkeys against DENV-1 challenge

All monkeys were challenged with a wild-type strain of DENV-1 2 months after the last booster. Viremia was measured by RT-PCR and virus isolation for 10 days after challenge (Table 2).

The mean viremia duration in *M. fascicularis* P64k monkeys was 1.5 and 3 days as determined by viral isolation and RT-PCR, respectively (Table 2). Even when the viral genome was recovered from the sera of both *M. fascicularis* control monkeys, virus isolation was completely negative in one of them. The *M. fascicularis* monkeys vaccinated with PD10 protein had no viremia measurable by viral isolation while viral RNA was present for 2 days only in one monkey.

The rhesus monkeys immunized with P64k protein showed 4 and 4.5 viremic days estimated by viral isolation and RT-PCR, respectively (Table 2). Viral isolation in the animals immunized with PD10 protein in Freund adjuvant was positive only at first day for monkey R0102, while monkey R4979 was fully protected. In addition, monkey R0102 showed viremia in 4 days as detected by RT-PCR. Conversely, there was no reduction in the duration of viremia (in terms of viral isolation or RT-PCR) in the rhesus monkeys immunized with the recombinant protein in aluminum hydroxide compared to the P64k control group.

The virus quantification as estimated by IFF assay was positive in the 76% of the serum samples showing viral isolation (Table 2). Serum samples from monkeys immunized with PD10 protein in Freund adjuvant (*M. fascicularis* and rhesus) were evaluated negative by IFF assay. In general, the group immunized with PD10 protein in aluminum showed similar viral titers compared to the P64k monkeys.

3.3. Antibody response after virus challenge

The antibody analysis demonstrated that all PD10 immunized animals developed a memory response after virus challenge, even those immunized with aluminum hydroxide. The IgG and neutralizing antibody titers increased more than four times between the sera collected at days 0 and 21 after virus challenge (Fig. 1 and Table 1). Anti-DENV-1 IgG and neutralizing antibodies raised slowly in the control animals, characteristic of a primary infection. The monkeys immunized with PD10 protein in Freund adjuvant showed higher neutralizing antibody titers on average compared to those immunized with aluminum hydroxide after challenge.

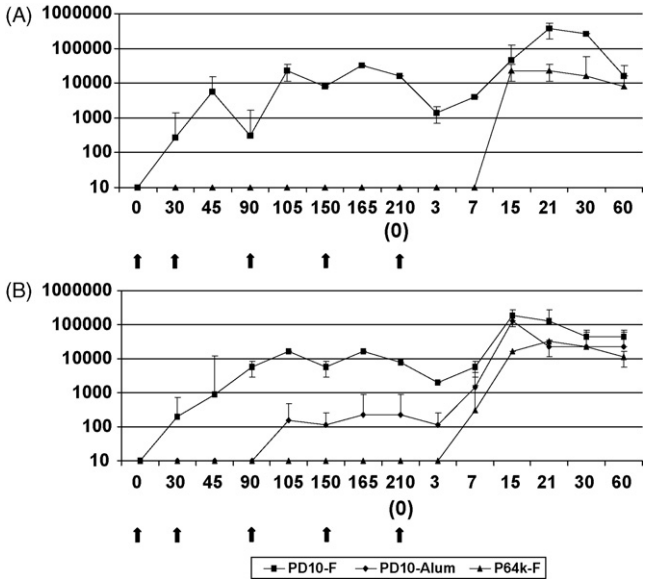

Fig. 1. Kinetics of the geometric mean titers (GMT) of IgG antibodies induced after immunization and challenge as detected by ELISA. *X* and *Y* axes represent schedule days and GMT, respectively. After challenge, days were renumbered (restarting at 0). Arrows indicate days of immunization and challenge. A: *M. fascicularis* monkeys; B: rhesus monkeys; F: Freund adjuvant; alum: aluminum hydroxide adjuvant.

Table 1
Neutralizing antibody titers against dengue 1 virus in monkeys after immunization and challenge

Monkeys	Group	Monkey #	Days after immunization and challenge								
			90[a] (3rd dose)[b]	105	150 (4th dose)	165	210 (0) (Ch)[c]	9	21	30	60
Macaca fascicularis	PD10 Freund	1426	<10	70	<10	410	<10	400	9,000	2,200	1,100
		1243	<10	640	10	1,000	54	250	3,400	1,300	10,000
	P64K Freund	6841	NT[d]	NT	NT	NT	<10	<10	<10	100	1,000
		4881	NT	NT	NT	NT	<10	<10	<10	31	68
Rhesus	PD10 Freund	R0102	<10	10	<10	90	<10	190	5,000	1,000	800
		R4979	<10	110	<10	210	<10	700	420	840	1,000
	PD10 Alum	R0101	<10	<10	<10	<10	<10	<10	100	200	250
		R0105	<10	<10	<10	<10	<10	280	580	150	280
	P64K Freund	R0104	NT	NT	NT	NT	<10	<10	560	2,900	280
		R0103	NT	NT	NT	NT	<10	<10	200	300	320

[a] No neutralizing antibodies were detected prior to this day.
[b] Dose.
[c] Challenge. The day of virus challenge was considered as day 0.
[d] Sample not tested.

Table 2
Viremia estimated by RT-PCR[a], viral isolation[b] and immunocytochemical focus-forming assay[c] after challenge with dengue 1 virus

Monkeys	Group	Monkey #	Days after challenge										Mean of days positive by VA[e]/RT-PCR
			1[d]	2	3	4	5	6	7	8	9	10	
Macaca fascicularis	PD10 Freund	1426											0/1
		1243											
	P64K Freund	6841			+(150)	+	+(87)						1.5/3
		4881											
Rhesus	PD10 Freund	R0102	+										0.5/2
		R4979											
	PD10 Alum[f]	R0101		+(12)	+(12)	+(37)	+(50)	+					4.5/5
		R0105		+(6)	+	+(75)	+(62)						
	P64K Freund	R0104		+(25)		+(1 237)	+(62)						4/4.5
		R0103		+(6)	+	+(50)	+(75)	+(300)					

[a] In gray, positive sera by RT-PCR. [b] +: dengue 1 virus isolation from serum. [c] Parentheses represent the focus-forming units/mL in serum. [d] The day of virus challenge was considered as day 0. [e] Viral isolation. [f] Aluminum hydroxide adjuvant.

4. Discussion

The antibody response after immunization demonstrated the immunogenic properties of the DENV-1 recombinant fusion protein emulsified in Freund adjuvant in *M. fascicularis* and rhesus monkeys. Irrespective of the animal model used, the neutralizing antibody titers obtained after the third immunization with PD10 protein in Freund adjuvant were similar to those reported previously for the evaluation of recombinant DENV-2 E domain III proteins in monkeys (Hermida et al., 2006; Simmons et al., 2006). In addition, neutralizing antibody titers induced were comparable to those induced by attenuated or DNA DENV-1 vaccines in humans or monkeys, respectively (Durbin et al., 2006; Raviprakash et al., 2000, 2003; Sun et al., 2003). However, as has been previously reported (Hermida et al., 2006; Simmons et al., 2006), the antibody titers declined rapidly over time and hence, the long-term safety of such a vaccine still needs to be evaluated.

After challenge with DENV-1, the rhesus monkeys were more susceptible to infection than *M. fascicularis*. Because of the fact that infective virus was not detected in one *M. fascicularis* control monkey, the mean viremia duration was dramatically reduced in this group as detected by viral isolation. However, according to the criterion of RNA and antibody response this control monkey (4881) was infected. In correspondence with viremia, the control rhesus monkeys developed earlier and higher antibody responses than *M. fascicularis* monkeys after DENV-1 inoculation. Nevertheless, the antibody response after PD10 immunization was higher in *M. fascicularis* monkeys supporting the usefulness of this model for evaluating DENV recombinant proteins.

Previous studies have shown an 80% protection from lethal challenge in mice immunized with PD10 protein (Hermida et al., 2004a). Similar to these results in mice, the monkeys immunized with PD10 in Freund adjuvant were almost fully protected. Viral isolation was negative in the majority of cases (monkey R0102 was the only one with a positive result 24 h after challenge). In addition, the monkeys immunized with PD10 in Freund adjuvant were at least partially protected if measured by RT-PCR. Even when viral RNA was detected in the animals immunized with Freund adjuvant, a remarkable reduction in the mean number of positive days was observed compared to the control group. While viral isolation reflects fully functional viral particles, the RT-PCR could also be positive by detecting viral genomes from non-infective immune complexes or defective virus. In addition, the absence of FFU in the serum from animals vaccinated with PD10 protein in Freund adjuvant indicates a limited viral replication even in the presence of ARN detection.

In addition, it was noteworthy that even as RT-PCR is considered to have a higher sensitivity compared to viral isolation (Rosario et al., 1998), some serum samples were negative by RT-PCR but the virus was isolated. Similar results were reported by Markoff et al.

(2002): DENV-1 was detected in one rhesus monkey inoculated with 6 log PFU up to day 2 by RT-PCR and up to day 3 by plaque titration (Markoff et al., 2002). The possible effect of polymerase activity inhibitors present in the serum samples, such as hemoglobin, could explain this observation (De Paula and Lopes da Fonseca, 2002). The presence of hemoglobin itself as a potential inhibitor depends on the quality of the blood extraction method and the quality of the serum preparation protocol, which explains why the RT-PCR reaction could eventually have been inhibited.

In contrast, PD10 in aluminum hydroxide failed to induce protective immunity in rhesus monkeys. Aluminum hydroxide with its combination of saponin and monophosphoryl lipid A showed lower immunogenicity and no protection from viremia compared with aluminum alone (Putnak et al., 2005).

In the absence of a good animal model for dengue, neutralizing antibodies are widely accepted as a surrogate marker of protective immunity, with PRNT titers $\geq$1:10 being considered as indicative of protective immunity (Edelman et al., 2003; Khanam et al., 2007). However, there was not a total correlation between neutralizing antibodies after immunization and protection from viremia. Neutralizing antibody titers decreased before the day of challenge in the majority of the animals and the monkey that showed positive values was not the fully protected one in terms of viral RNA detection. A similar observation has been previously reported (Endy et al., 2004; Guzmán et al., 2003; Hermida et al., 2006; Sun et al., 2006) and suggests either that very low levels of neutralizing antibodies (<1:10) are sufficient for protection or that other immune mechanisms such as cell-mediated immunity contribute to protect from viremia. One of the current research priorities in dengue vaccine is the definition of markers of protection allowing a better evaluation of vaccine candidates.

The analysis of the anamnestic response showed a rapid increase in the antibody titers after challenge for the animals immunized with PD10 protein in Freund adjuvant, indicating a successful priming by the recombinant protein. Even when vaccinated animals were protected, the anamnestic antibody response indicates that there was virus replication at least confined to the injection site. However, anamnestic antibody response and RNA detection in serum have been observed in animals inoculated and challenged with homologous viruses (Bernardo et al., 2008). In addition, several vaccine candidate evaluations have shown anamnestic responses after challenge for animals fully protected in terms of viremia development (Blair et al., 2006; Guzmán et al., 2003; Markoff et al., 2002; Putnak et al., 2005; Raviprakash et al., 2000).

4.1. Conclusions

The recombinant protein that includes the domain III of DENV-1 fused to the P64k protein from *N. meningitidis* in Freund adjuvant is immunogenic and protective in non-human primates. In addition, both *M. fascicularis* and rhesus *monkeys* are suitable for evaluating vaccine candidates against DENV. However, because aluminum hydroxide failed to induce a protective immune response, additional studies for testing other adjuvants licensed for humans are required before clinical trials. In addition, a larger number of animals need to be tested.

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