ORIGINAL ARTICLE

Screening for immune response against Dengue virus in Vietnamese non-human primates: implications for vaccine developers

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One of the major problems faced for the development of a vaccine against Dengue virus is the lack of a suitable animal model. Although non-human primates do not show overt signs of disease, these animals develop viremia after the infection and are the best model to evaluate vaccine candidates against this pathogen. However, for that purpose, the screening of all animals is mandatory to discard those with previous natural immunity. The most common technique used in the screening is the plaque reduction neutralization test (PRNT). However, most recent studies points to the cell-mediated immunity (CMI) as an important player in the process of controlling Dengue virus (DENV) infections. Here we presented the results from the screening of 55 rhesus monkeys housed in an animal breeding facility at Quang Ninh province, Vietnam. We evaluated the neutralizing antibody response by PRNT and determined the levels of interferon γ (IFNγ)-secretion after the viral stimulation of monkey-peripheral blood mononuclear cells, by enzyme-linked immunosorbent assay (ELISA). We found no correspondence between PRNT and IFNγ-ELISA. In fact, 19 animals were positive only by IFNγ-ELISA. Moreover, to study the protective capacity of the CMI detected, three animals with positive response by IFNγ-ELISA and negative by PRNT were inoculated with an infective preparation of DENV-3 and, as a result, no viremia was detected during 10 days after the challenge. This fact points to the importance of screening non-human primates through a CMI assay together with PRNT. This procedure should discard those false-negative cases which would be protected after the viral challenge in the immunization schedule.

Dengue virus (DENV) is a flavivirus consisting of four serotypes named DENV-1, DENV-2, DENV-3 and DENV-4. These viruses are transmitted to humans through the bite of infected-mosquitoes. An estimated of 390 million infections and 96 million symptomatic cases of dengue occurs annually.[1]

The lack of a suitable animal model has hampered the progress of a dengue vaccine. Although several small-animal models have been developed over the years, none of them reproduces all the signs of the disease. Several species of non-human primates have been evaluated for DENV infection.[2] These studies showed that these animals are susceptible to develop levels of viremia depending of the specie and serotype. Moreover, these animals generate a consistent antibody response, but do not show overt signs of disease.

To reliably evaluate a vaccine candidate against Dengue in non-human primates, it is critical to screen all animals looking for previous immunity against Dengue or another flavivirus. Usually, the screening consists in performing a Dengue immunoglobulin G (IgG)-enzyme-linked immunosorbent assay (ELISA) and a plaque reduction neutralization test (PRNT) and those animals without a positive humoral immune response are included in the study. However, most recent studies points to the cellular arm of the adaptive immune system as an important player in the process of controlling DENV infections.[3–5] These evidences advise that the cell-mediated immunity (CMI) must be taken into account for the screening of non-human primates, since high levels of protection can be due to previous DENV-immunity and not to the evaluated vaccine candidate.

In this work, we present a preliminary evaluation of the influence of the cellular immune response (measured through the interferon γ (IFNγ) secretion by DENV-stimulated peripheral blood mononuclear cells (PBMC), detected by ELISA) in the viremia developed by Rhesus monkeys after an experimental infection with DENV-3. Moreover, we describe the results from serological studies of these animals and discuss the implications of the differences between PRNT and IFNγ-ELISA as techniques for selecting animals for a vaccination study. Finally, we analysed our seroprevalence results in the epidemiological context in the Asiatic region.

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Table 1 Percentage of viral plaques reduction in African green monkey kidney (VERO) cells by monkey sera

Mono	DENV-1	DENV-2	DENV-3	DENV-4	Mono	DENV-1	DENV-2	DENV-3	DENV-4
1	19.71	**52.21**	0	0	29	41.61	4.41	42.86	21.05
2	0	19.12	3.57	0	30	41.61	48.53	35.71	28.57
3	8.76	48.53	39.29	28.57	31	27.01	0.74	35.71	6.02
4	0	19.12	17.86	28.57	32	**52.55**	15.44	35.71	32.33
5	1.46	22.79	21.43	39.85	33	41.61	37.50	0	0
6	0	33.82	39.29	21.05	34	34.31	37.50	**57.14**	2.26
7	12.41	44.85	39.29	6.02	35	19.71	22.79	21.43	6.02
8	**52.55**	33.82	**64.29**	32.33	36	41.61	0.74	35.71	28.57
9	34.31	26.47	32.14	17.29	37	34.31	22.79	53.57	2.26
10	12.41	26.47	35.71	36.09	38	30.66	11.76	35.71	9.77
11	1.46	8.09	28.57	36.09	39	16.06	30.15	35.71	9.77
12	0	0	**53.57**	28.57	40	16.06	26.47	3.57	0
13	23.36	33.82	21.43	21.05	41	45.26	**55.88**	32.14	0
14	41.61	22.79	25.00	6.02	42	8.76	4.41	25.00	2.26
15	5.11	0	21.43	0	43	19.71	30.15	**57.14**	0
16	16.06	4.41	39.29	0	44	48.91	0	21.43	0
17	**56.20**	30.15	17.86	9.77	45	37.96	26.47	3.57	0
18	41.61	19.12	10.71	9.77	46	41.61	11.76	**53.57**	2.26
19	23.36	37.50	28.57	21.05	47	23.36	22.79	28.57	6.02
20	23.36	30.15	17.86	9.77	48	37.96	0	25.00	0
21	37.96	33.82	25.00	0	49	30.66	26.47	0.00	24.81
22	19.71	30.15	10.71	0	50	34.31	26.47	39.29	24.81
23	5.11	44.85	28.57	0	51	**52.55**	41.18	**53.57**	36.09
24	27.01	22.79	25.00	17.29	52	12.41	15.44	39.29	2.26
25	34.31	0	0.00	6.02	53	30.66	22.79	**50.00**	39.85
26	19.71	26.47	35.71	32.33	54	37.96	37.50	28.57	9.77
27	37.96	4.41	21.43	0	55	41.61	37.50	21.43	21.05
28	19.71	15.44	32.14	0	Percentage[a]	7.3	3.6	12.7	0

Abbreviation: DENV, dengue virus.
[a]Percentage of animals with more than 50% viral plaques reduction per serotype. Values ⩾ 50% are in bold. The unique serum dilution tested was 1:10.

RESULTS

Humoral immunity against Dengue

An amplified sandwich ELISA system was used to detect anti-DENV IgG antibodies. None of the animals showed antibody titers higher or equal to 1:100 against the four Dengue serotypes. However, 20% (11 out of 55) monkeys exhibited neutralizing antibody titers ($\geqslant$1:10) at least against one serotype (Table 1). For each serotype the percentages of animals with neutralizing antibodies were 7.3% (4/55) for DENV-1, 3.6% (2/55) for DENV-2 and 12.7% (7/55) for DENV-3. Only 3.6% (2/55) were positive for both DENV-1 and DENV-3. None of the animals showed neutralizing antibodies against DENV-4 only. Note that 44/55 (80.0%) were negative for PRNT$_{50}$.

Cellular immunity against Dengue measured by IFNγ-secretion after viral stimulus

PBMC from monkeys were stimulated with infective DENV to measure IFNγ secretion by ELISA (Figure 1a). As a result, 26/55 (47.3%) animals showed a positive response against one or more Dengue serotypes. In accordance with PRNT results, the positive response against DENV-3 was predominant (22/55; 40%) while a 30.1% of animals were positive for DENV-1 (Figure 1b). In addition, 25.4% were positive for DENV-2 and DENV-4. The 52.7% (29/55) of the animals were negative to all serotypes.

Viral challenge

To corroborate the results obtained in PRNT and IFNγ- ELISA, six animals were challenged by subcutaneous route with 10^3 p.f.u. of

DENV-3. Three of them were negative by both techniques while the others were negative by PRNT and positive by IFNγ-ELISA. Two of the last ones had high levels of IFNγ-secretion after the stimulation with DENV-3 (monkey #31: 1346.7 pg ml^{-1}; monkey #33: 1933.5 pg ml^{-1}), whereas the rest one showed a low level (monkey #22: 738.3 pg ml^{-1}). Blood was collected daily during 10 days to detect viremia. The presence of virus in serum samples was determined by plaque assay, inoculating 0.1 ml of undiluted serum onto VERO cell. As shown in Table 2, animals that were negative by PRNT and IFNγ-ELISA developed 3 days of viremia and an average viral load of $10^{1.7}$ p.f.u. ml^{-1}. In turn, monkeys negative by PRNT and positive by IFNγ-ELISA were completely protected.

DISCUSSION

In this study, we analyze the immune response against Dengue virus in 55 Rhesus monkeys captured on Reu island, from the Quang Ninh province, Vietnam, in 2012. We employed, in a first step, the PRNT, which is the technique most used in the screening of primates for immunization schedules and assessments of vaccine candidates against DENV.[6–9] The results suggest that 80% of the animals from the island had not had prior contact with Dengue virus as they had no detectable neutralizing humoral response.

In a second step, we conducted an assay of IFNγ-secretion detected by ELISA, after PBMCs being *in vitro*-stimulated with the four DENV-serotypes. Unlike PRNT, which measures levels of circulating neutralizing antibodies, IFNγ-ELISA detects memory cellular immune response which is activated after the *in vitro* stimulation with the virus.
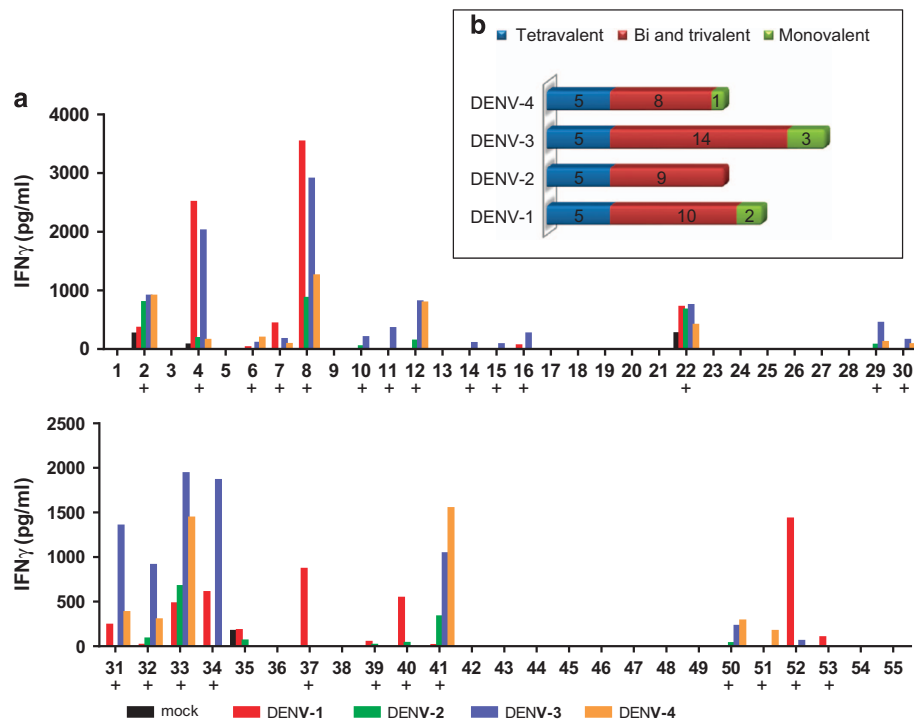
Figure 1 IFNγ secretion of PBMCs stimulated with DENV. (**a**) PBMCs from 55 animals were stimulated *in vitro* with each DENV produced in VERO cells and IFNγ concentrations in the resulting culture supernatants were determined by ELISA. Mock: supernatant from non-infected VERO cells. **+:** Positive response was considered when the IFNγ concentration value in the stimulated PBMCs was twice or higher than the IFNγ concentration in cells without viral stimulus. (**b**) Number of monkeys positive to one serotype (monovalent), to two-three serotypes (bi-trivalent) and to four serotypes (tetravalent).

Table 2 Viremia levels after experimental infection with DENV-3

Monkey #	Days after challenge (log p.f.u. ml^{-1})[a]										Immune status
	0	1	2	3	4	5	6	7	8	10	
33											Negative by PRNT, DENV-positive for IFNγ-ELISA
22											
31											
18			1.3	2.1	1.8						Negative for both techniques
25			1.6	2.3	2.2						
47			1.7	1.6	–	1.0					

Abbreviations: DENV, dengue virus; ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon γ; PRNT, plaque reduction neutralization test.
[a]Six animals were infected by subcutaneous route with 10^3 p.f.u. of DENV-3. Blood was collected daily during 10 days to detect viremia. The presence of virus in serum samples was determined by plaque assay, inoculating 0.1 ml of undiluted serum onto VERO cells.

Unexpectedly, 40% of the animals showed a positive IFNγ response following stimulation with DENV-3 and 47.3% showed a positive response on stimulation with at least one of the other serotypes.

In general, we found no correspondence between both techniques; in fact, only seven animals showed neutralizing antibody response and IFNγ secretion, four animals resulted positive only by PRNT and 19 were positive only by IFNγ-ELISA. These results might be explained by the fact that after a natural infection, monkeys developed a low-level viremia and consequently, a low humoral immune response that declined in a few weeks. However, the memory T-cell response induced by the natural infection can be activated after an *in vitro* stimulation of PBMCs thus secreting detectable levels of IFNγ.

Some studies suggest that the stimulation of PBMCs with infectious viruses can lead to IFNγ response in the culture supernatant.[10] In accordance, DENV-related flavivirus such as yellow fever virus can trigger TLR2, 7, 8, and 9 on dendritic cells[11] and it is plausible that this

could result in innate cytokine production that in turn, could stimulate IFNγ responses by T cells in a non-virus-specific fashion. However, there are several published studies in which mouse-splenocytes and monkey-PBMCs from non-immunized groups did not secrete IFNγ after the *in vitro* stimulation with infectious DENV,[3,12,13] suggesting specific antiviral responses. Future studies using splenocytes and/or PBMCs *in vitro*-stimulated with overlapping peptides and infectious DENV will clarify this issue.

To study the protective capacity of the CMI detected, three animals with positive response by IFNγ-ELISA and negative by PRNT, as well as three with negative responses by both techniques, were inoculated with an infective preparation of DENV-3. The first animals were protected while the others developed measurable viremia, during 3 days. This study evidences that cell-mediated immunity is able to protect animals from viral challenge, even without the presence of neutralizing antibodies detectable at the date of challenge. The

erroneous inclusion of DENV-immune animals in an immunization schedule to evaluate a vaccine candidate against this pathogen could lead to wrong estimates of protection levels in vaccinated subjects or low viremia in placebos and hence, to misreading the results.

Recent studies point to the cellular arm of the adaptive immune system as an important player in the process of controlling DENV infections. Several different mouse experiments have demonstrated that CD8+ and even CD4^{+} T cells contribute to protection against DENV, decreasing the severity of disease manifestations or reducing the viral load in blood and other organs.[5,14,15] Moreover, our group has developed CMI-based vaccine candidates using the recombinant capsid protein from DENV.[3,12] Recently, these authors reported that the tetravalent formulation based on recombinant nucleocapsid-like particles from dengue viruses induces in both mice and monkeys an IFNγ-secreting cell response that significantly reduces viral load after challenge without the contribution of neutralizing antibodies.[16]

In our study, animals were not screened for another flavivirus such as Japanese encephalitis or Zika. However, if the detected IFNγ response was due to a DENV cross-reactive response induced by any flavivirus, the implication of our findings on DENV vaccine evaluation remains.

Two other points deserve discussion based on our results. First, the fact that although none of the animals showed detectable antiviral antibody titers by ELISA, 20% of the animals had neutralizing antibodies. Differences between the results obtained by both techniques (ELISA and PRNT) could be due to the initial dilution of sera used in the ELISA (1:100) which was 10 times higher than that evaluated in PRNT. In our experience, higher concentrations of sera in monkey IgG-ELISA increase unspecific interactions. Another possible explanation for these different results should be the viral strains used in each case; for ELISA we used neuro-adapted strains from infected mouse brain and for PRNT we used viral strains obtained from VERO cell culture.

Moreover, as we hypothesized above, DENV-natural infections in monkeys induce low neutralizing humoral response. Although it is known that these animals are involved in the natural cycle of transmission, only few studies describe DENV-seroprevalence in non-human primates. Kato et al.[17] described a study in 100 cynomolgus monkeys from an animal breeding facility in the Philippines. A high incidence of DENV was detected since 21 animals were IgM positive and 19 monkeys resulted IgG positive.[17] However, PRNT identified only nine positive sera and only one sample had neutralizing antibody titers higher that 1:100. In accordance with our results, there were animals (3) positive by PRNT while negative by IgG-ELISA. This study also supports the idea that there is no necessarily straightforward relationship between results from IgG-ELISA and PRNT. Anyway, taking into account our findings, we should expect that Kato et al. would find more positive animals if a CMI assay were used.

Second, the immune response detected in our study was predominantly directed against DENV-3, which suggests that this serotype circulated in Reu Island. According to Arima et al.,[18] in 2012 Vietnam reported 86 026 clinical Dengue cases. All four serotypes were detected, with DENV-1 being the most common. However, in the same year, 9952 clinical dengue cases were reported in the neighbor Lao People's Democratic Republic, which doubled the number of cases reported in 2011. Although all four serotypes circulated, the predominant serotype was DENV-3. In general, all four serotypes were circulating in the Asian region in 2012, being serotypes 1 and 3 the most common. It is important to highlight that these serotypes have the highest percentage of identical amino acids (78.4%) in the envelope protein.[19]

In conclusion, our results suggest that for the evaluation of vaccine candidates in non-human primates it is not enough to screen animals by testing the immune response against Dengue only by ELISA and PRNT, as is done regularly. Besides, a CMI assay–stimulating with viral antigens–should be carried out to discard false-negative cases, which would be protected after the viral challenge in the immunization schedule.

METHODS

Cells and viruses

African monkey kidney cells (VERO) were received from the National Institute for Biological Standards and Control (NIBSC; NIBSC accession number: 011038). Cells were grown at 37 °C in Eagle's minimal essential medium supplemented with 10% fetal bovine serum.

The following virus strains were used for antibody detection and CMI: Hawaii (DENV-1), New Guinea C (DENV-2), H-87 (DENV-3) and H241 (DENV-4).[20] For CMI viruses were concentrated by centrifugation at 80 000 g, 4 h at 4 °C, homogenizing the resulting pellet in 1 ml of phosphate-buffered saline (Gibco, Paisley, UK). A mock preparation was prepared from the supernatant of uninfected VERO cells following a similar procedure. DENV-1 strain Jamaica CVI 1636 strain, DENV-2 SB8553 strain, DENV-3 Nicaragua 163/94 strain and DENV-4 Dominica 814669 strain were used for the PRNT. For the challenge study in monkeys, a viral stock was prepared with DENV-3 Nicaragua 163/94 strain.[21]

Animals

55 young monkeys (*Macaca mulatta*) were obtained from the Reu Island located in the northern province of Quang Ninh, Vietnam. The island is administered by the Centre for Research and Production of Vaccines and Biologicals (Polyvac), it has a total area of 22 hectares and a dense forest where living about a thousand of monkeys.

Animal group average were 18.4 months old and weight 2.09 kg and were 27 male and 28 female (Table 3). The study was carried out in Polyvac facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Animals were maintained throughout the study in individual cages that permitted the evaluation and patterns conduct according to their species, size, age and sex. They were fed with fruits and vegetables and water was available *ad libitum*. All immunizations and blood extractions were performed using ketamine hydrochloride, 10 mg kg^{−1} body weight, and all efforts were made to minimize suffering.

Monkeys challenge and viremia detection

Six monkeys were subcutaneously inoculated in the upper arm with 10^3 p.f.u. of DENV-3. Blood was collected daily for 10 days to detect viremia. The presence of virus in serum samples was determined by direct plaque formation on VERO cells as previously described.[22]

Measurement of humoral immune response

The anti-DENV IgG antibodies induced by immunization were monitored by ELISA as previously described by Gil et al.,[3] Titers were defined as the dilution of serum giving twice the absorbance value of the negative control serum.

The functionality of the antibodies was measured by PRNT[23] in VERO cells. The unique serum dilution tested was 1:10. The number of viral plaques by serotypes was: 13.7 (DENV-1), 13.6 (DENV-2), 14 (DENV-4) and 13.3 (DENV-4). The monoclonal antibody 4G2 was used as positive control.[24]

Measurement of cellular immune response

Monkey blood was obtained and PBMCs were isolated as previously described.[21] Cells were washed twice with phosphate-buffered saline 2% fetal bovine serum (PAA Laboratories, Ontario, Canada) and homogenized at 2×10^6 cells per ml in Roswell Park Memorial Institute 1640 medium (Sigma Aldrich) supplemented with 100 U ml^{−1} penicillin, 100 μg ml^{−1} streptomycin (Gibco, UK), 2 mM glutamine (Gibco, UK), 5×10^{-5} M 2-mercaptoethanol (Sigma, St Louis, MO, USA) and 5% fetal bovine serum. Finally, 2.5×10^5 cells per well were cultured in 96-well round bottom plates

Table 3 Features of macaca monkeys from Reu island, Quang Ninh province, Vietnam

Monkey number	Weight (Kgs)	Sex (F or M)	Age (month)	Monkey number	Weight (Kgs)	Sex (F or M)	Age (month)
1	1.6	M	15	29	3.8	M	24
2	2.5	M	20	30	3.7	M	24
3	1.8	F	16	31	1.7	M	18
4	2.3	M	18	32	2.5	F	20
5	2	F	20	33	2	F	18
6	1.8	F	18	34	2.1	M	18
7	2.4	F	20	35	2.5	M	24
8	2.6	F	20	36	1.8	F	18
9	2.2	M	20	37	2	F	18
10	1.8	M	16	38	1.8	F	18
11	2.4	F	18	39	2	F	18
12	2	M	18	40	2.7	F	24
13	2.4	M	20	41	1.7	M	16
14	2	F	20	42	1.5	M	16
15	2.1	F	18	43	1.4	F	16
16	1.6	M	16	44	2.5	F	18
17	1.7	M	16	45	2.5	F	20
18	2.5	F	18	46	2.6	M	24
19	2.1	F	18	47	2	F	18
20	2.3	F	20	48	2.4	F	24
21	2	M	18	49	1.7	F	18
22	2	F	18	50	1.6	M	15
23	3	M	20	51	1.7	F	16
24	2	M	18	52	2	M	16
25	2	F	18	53	1.6	M	16
26	1.6	F	18	54	1.8	M	16
27	1.6	M	18	55	1.4	M	14
28	1.7	M	16	Average	2.09 ± 0.48	27 M, 28 F	18.4 ± 2.45

Abbreviations: M, male; F, female.

and stimulated with the mock preparation or DENV at multiplicity of infection (MOI) of 0.5.

Concanavalin A (Sigma) was used as a positive control. In all experiments two wells were plated for each antigen. After 4 days of culture, culture supernatants were collected and stored at −80 °C. The culture supernatants were analyzed in duplicate for INFγ concentrations by ELISA using monoclonal antibody pairs (Mabtech INFγ; Nacka Strand, Sweden) and the protocol recommended by the manufacturer. The optical density at 492 nm in cells stimulated with mitogen was used to normalize data. Positive response was considered when the IFNγ concentration value in the stimulated PBMCs was twice or higher than the IFNγ concentration in cells without viral stimulus.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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