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Attenuation and immunogenicity of recombinant yellow fever 17D-dengue type 2 virus for rhesus monkeys

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Abstract

A chimeric yellow fever (YF)-dengue serotype 2 (dengue 2) virus was constructed by replacing the premembrane and envelope genes of the YF 17D virus with those from dengue 2 virus strains of Southeast Asian genotype. The virus grew to high titers in Vero cells and, after passage 2, was used for immunogenicity and attenuation studies in rhesus monkeys. Subcutaneous immunization of naive rhesus monkeys with the 17D-D2 chimeric virus induced a neutralizing antibody response associated with the protection of 6 of 7 monkeys against viremia by wild-type dengue 2 virus. Neutralizing antibody titers to dengue 2 were significantly lower in YF-immune animals than in YF-naive monkeys and protection against challenge with wild-type dengue 2 virus was observed in only 2 of 11 YF-immune monkeys. An anamnestic response to dengue 2, indicated by a sharp increase of neutralizing antibody titers, was observed in the majority of the monkeys after challenge with wild-type virus. Virus attenuation was demonstrated using the standard monkey neurovirulence test. The 17D-D2 chimera caused significantly fewer histological lesions than the YF 17DD virus. The attenuated phenotype could also be inferred from the limited viremias compared to the YF 17DD vaccine. Overall, these results provide further support for the use of chimeric viruses for the development of a new live tetravalent dengue vaccine.

Key words
- YF 17D virus recombinants
- Dengue vaccine
- Immunogenicity
- Attenuation
- Rhesus monkeys

Introduction

The genus Flavivirus consists of 70 members, several of which cause human illnesses with the most important being yellow fever (YF), Japanese encephalitis, tick-borne encephalitis, and dengue type 1-4 viruses. Dengue viruses have spread throughout the tropics, a fact that was accompanied by an increasing frequency of the more severe forms of this disease, i.e., dengue hemorrhagic fever and dengue shock syndrome (1). Internationally approved vaccines are available only for YF (live 17D vaccine) (2), and tick-borne encephalitis and Japanese encephalitis (inactivated vaccines) (3). Live attenuated vac-

cine candidates for dengue viruses have been developed using serial passages in cultured vertebrate cells and tested in humans (4-8), but so far none has been licensed.

Since the establishment of the prototype flavivirus genome structure and expression (9), recombinant DNA technology has been an alternative for flavivirus vaccine development (for a review, see Ref. 10). In particular, a strategy first developed by Bray and Lai (11) has been used by several groups to create new chimeric flaviviruses through the exchange of the viral envelope proteins (for a review, see Ref. 12). In this context, the YF 17D virus, one of the most effective and safest vaccines available and therefore very attractive as a live carrier, was used for insertion of the premembrane/envelope (prM/E) genes of several dengue viruses (13-17), resulting in attenuated and immunogenic chimeras. Here, we describe the attenuation, immunogenicity and protective ability of a chimeric 17D-dengue serotype 2 virus in rhesus monkeys.

Material and Methods

Cells and viruses

Vero cells (ATCC, CCL 81) were maintained in 199 medium with Earle's salts (E199) buffered with sodium bicarbonate and supplemented with 5% fetal bovine serum and antibiotics.

The wild-type dengue 2 (D2) 44-2 strain has been described elsewhere (13). It originated from a human case of dengue fever and belongs to the group of D2 Southeast Asian genotype viruses more recently introduced in the Americas (18). YF 17DD is a live attenuated virus used in the YF vaccine manufactured by Bio-Manguinhos, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ, Brazil.

The 17D-D2 virus was obtained after transfection of Vero cells with *in vitro* transcribed full-length RNA (13). The supernatant resulting from the transfected culture was harvested when a cytopathic effect was evident. This stock was titrated and used to infect two T175 flasks of Vero cells at a multiplicity of infection of 0.002. The supernatant was harvested 7 days later when cytopathic effect was pronounced, supplemented with 10% sorbitol as a stabilizer, aliquoted and frozen at -70ºC. This virus was used for all experiments described below at passage 2. All virus stocks or monkey serum samples were titrated by plaque formation on Vero cell monolayers using 6-well plates and carboxymethylcellulose as overlay (13).

RT/PCR and sequencing

Viral suspensions were used for RNA extraction with Trizol LS (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA). The extracted RNA was used as template for cDNA synthesis with YF or Den-specific synthetic oligonucleotide primers (GeneAmp RNA PCR Core Kit, Perkin-Elmer, Boston, MA, USA). Primers were designed on the basis of YF 17D strain sequence (Gene bank accession number X03700) and the D2 NGC strain sequence (M29095). PCR products were gel purified (Qiaquick gel extraction kit, Qiagen, Hilden, NDRW, Germany) and sequenced using the ABI PRISM dye terminator cycle sequencing core kit and an ABI 3100 instrument (Applied Biosystems, Foster City, CA, USA).

Monkeys

All rhesus monkeys (*Macaca mulatta*) were obtained from the Primatology Department of the Center for Laboratory Animals (CECAL) of FIOCRUZ. Prior to inoculation, all monkeys were shown to be free of YF and dengue-neutralizing antibodies by the plaque reduction neutralization titer (PRNT) assay. Each animal was kept in a separate cage under controlled environmental conditions (temperature of 20-22ºC, rela-

tive humidity of ~60% and 12 h of artificial light and 12 h of darkness). Animals were fed twice daily with monkey chow supplemented with fresh fruits and were allowed water *ad libitum*.

Studies were carried out according to a protocol approved by the Institutional Committee for Experimentation and Care of Research Animals (CEUA-FIOCRUZ: P0112/02).

Immunogenicity studies: Experiment 1

A total of 12 rhesus monkeys, 2 females and 10 males weighing 3,400 to 7,240 g, were divided into 4 groups of 3 animals each and used in the experiment. The overall design of immunogenicity experiment 1 is given in Table 1. Group 1 received a subcutaneous (*sc*) dose of the chimeric 17D-D2 virus at day 30 and was challenged with 4.85 $\log_{10}$ plaque-forming units (PFU) of D2 44-2 virus by the *sc* route at day 60. Group 2 received an *sc* dose of YF 17DD vaccine (4.3 $\log_{10}$ PFU) at day 0 and a similar dose of chimeric 17D-D2 virus 30 days later (day 30). The animals were challenged as above on day 60. Group 3 received the YF 17DD vaccine only at day 30 and was challenged with D2 44-2 virus on day 60. Group 4 received solely the challenge virus at day 60. Samples for viremia measurements were collected on days 1-8 after inoculation of YF 17DD virus, 1-8 days after 17D-D2 virus and 1-10 days after D2 44-2 virus. All viremias were assayed by plating serum samples onto Vero cells (undiluted and at 1:30 and 1:300 dilutions). Blood samples were taken at days 0, 15, 30, 45, 60, 75, and 90 for measuring the antibody response to D2 and YF by the PRNT assay.

Immunogenicity studies: Experiment 2

This experiment was designed to extend our observations on the immunogenicity of the 17D-D2 virus and the influence of YF pre-immunity on the protective efficacy of the chimeric virus. A total of 12 male rhesus monkeys weighing 2,110 to 3,260 g were divided into 3 groups of 4 animals each (Table 2). Group 1 was immunized with YF 17DD at day 0, re-immunized with 17D-D2 virus at day 30 and challenged with D2 44-2 virus 60 days later (day 90). Group 2 was immunized with YF 17DD at day 0, re-immunized with 17D-D2 virus at day 120 and challenged with D2 44-2 virus at day 180. Group 3 was immunized with 17D-D2 virus at day 0 and challenged with D2 44-2 virus at day 60. Blood samples for the analysis of seroconversion by PRNT were taken on day 0 (all pre-immune samples), day 30 (group 1 with YF 17DD and groups 1-3 after challenge inoculation with 44-2 virus) or day 60 (groups 1-3 with 17D-D2) after the respective viral inoculation. For group 2, antibody to YF was also measured at day

Table 1. Design of Experiment 1 on the immunogenicity of 17D-D2 virus in naive and YF-immune rhesus monkeys.

Group	1st inoculation (0-30 days)			2nd inoculation (31-60 days)			3rd inoculation (61-90 days)		
	Virus	Viremia (days)[a]	Seroconversion (days)[b]	Virus	Viremia (days)[a]	Seroconversion (days)[b]	Virus	Viremia (days)[a]	Seroconversion (days)[b]
1	-	-	-	17D-D2	1-8	45, 60	D2 44-2	1-10	75, 90
2	17DD	1-8	0, 15, 30	17D-D2	1-8	45, 60	D2 44-2	1-10	75, 90
3	-	-	-	17DD	1-8	45, 60	D2 44-2	1-10	75, 90
4	-	-	-	-	-	-	D2 44-2	1-10	75, 90

[a]Serial bleedings from days 1-8 (17DD) or 1-10 (17D-D2 and D2 44-2) after inoculation; [b]days from the beginning of experiment; YF 17DD virus dose: 4.3 $\log_{10}$ PFU; 17D-D2 virus dose: 4.87 $\log_{10}$ PFU; D2 44-2 virus dose: 4.85 $\log_{10}$ PFU.

120 prior to the administration of the 17D-D2 virus. For all groups the interval between immunization with 17D-D2 virus and challenge was 60 days. Viremia samples were collected on days 2, 4, and 6 after YF 17DD immunization, days 1-8 after 17D-D2 and days 1-10 after D2 44-2 challenge.

Viral neutralization

PRNT assays were carried out in Vero cells in 6-well plates as described elsewhere (19). The neutralizing antibody titer or 50% PRNT ($PRNT_{50}$) was identified as the highest serum dilution that reduced the number of virus plaques by 50% or more. The challenge viruses used in the PRNT were YF 17DD and D2 44-2.

Monkey neurovirulence test

This test was performed in two groups of 10 captive-bred healthy rhesus monkeys composed of 12 males and 8 females (2,430 to 3,600 g). Methods for intracerebral virus inoculation (5.05 log_{10} PFU/mL), measurements of viremia and seroconversion, clinical observations, autopsy, and histological examination have been described (20,21).

Statistical analysis

Means and standard deviations were calculated for clinical and combined histological scores. When there was a suggestion that the data were asymmetrical or variances were not homogeneous, the Kruskal-Wallis non-parametric test was performed. Differences were considered to be statistically significant when P was 0.05 or less. Statistical analyses were done using the software Stata 7.0 (Stata Corporation, College Station, TX, USA, 2002).

Results

Establishing the wild-type dengue 2 challenge model

In this preliminary experiment, we analyzed the infectivity of a wild-type D2 virus to be used to challenge monkeys. Five rhesus monkeys were inoculated with a single *sc* dose of 5.0 log_{10} PFU of D2 44-2 virus. Monkeys were bled at day 0 and thereafter for 10 consecutive days. Viremia, assayed by plaque titration in Vero cell monolayers, was detectable from days 1 to 8 in 3 animals and on days 1 through 6 in the remaining two. The magnitude of viremia ranged from 0.88 to 2.87 log_{10} PFU/mL (data not shown). Given the consistency of the D2 44-2 virus in generating measurable viremias in all animals we reasoned this would be an appropriate virus to be used to challenge monkeys after immunization with the 17D-D2 virus.

Immunogenicity of the 17D-D2 virus

In order to examine the immunogenicity

Table 2. Design of Experiment 2 on the immunogenicity of 17D-D2 virus in naive and YF-immune rhesus monkeys.

Group	1st inoculation			2nd inoculation			3rd inoculation		
	Virus	Viremia (days)[a]	Seroconversion (days)[b]	Virus	Viremia (days)[a]	Seroconversion (days)[b]	Virus	Viremia (days)[a]	Seroconversion (days)[b]
1	17DD	2, 4, 6	0, 30	17D-D2	1-8	90	D2 44-2	1-10	120
2	17DD	2, 4, 6	0, 120	17D-D2	1-8	180	D2 44-2	1-10	210
3	-	-	-	17D-D2	1-8	60	D2 44-2	1-10	90

[a]Bleedings at days 2, 4, and 6 after YF 17DD innoculation or serial bleedings from days 1 through 8 or 1 through 10 after inoculation with 17D-D2 or D2 44-2 viruses, respectively; [b]days from the beginning of experiment; YF 17DD virus dose: 4.78 log_{10} PFU; 17D-D2 virus dose: 5.1 log_{10} PFU; D2 44-2 virus dose: 5.03 log_{10} PFU.

of the chimeric virus we carried out two separate experiments, both including groups of flavivirus-naive and YF-immune rhesus monkeys.

In experiment 1, 12 rhesus monkeys were divided into 4 groups of 3 each. Groups 2 and 3 were given one human dose of YF 17DD vaccine (at day 0 and day 30, respectively; Table 1). Thirty days later, groups 1 (naive) and 2 were given an equivalent dose of 17D-D2 virus. At day 60 all 12 animals were challenged *sc* with 4.87 log_{10} PFU of D2 44-2 virus. The data on seroconversion and viremia after challenge are shown in Table 3.

In group 1, 2 of 3 flavivirus-naive animals that received the 17D-D2 virus were protected from challenge. Animal 151 had a lower neutralizing antibody response to D2 (1:108 and 1:63, at 15 and 30 days post-infection (*pi*), respectively) compared to the other 2 animals, and was not protected (Table 3). Animals L18 and M22 had higher PRNT (1:731 and 1:1310, respectively) and were protected, as evidenced by the absence of viremia (Table 3). The 3 YF-immune monkeys (group 2) developed measurable neutralizing antibodies to D2 after immunization with the 17D-D2 virus but only one monkey (151^A), which had a relatively high PRNT of 1:407, did not show viremia after challenge with 44-2 virus. All 6 animals in groups 1 and 2 showed a boost type response after challenge with wild-type D2 virus (Table 3).

As expected, the group 3 YF-immune animals had no antibodies to D2 until after challenge with D2 44-2 virus and accordingly all 3 animals developed measurable post-challenge viremia of over 3 log_{10} PFU/mL (Table 3). The group 4 naive monkeys showed no antibodies to YF or D2 viruses and developed viremia after challenge with wild-type D2 virus (Table 3).

In the second experiment we expanded the number of flavivirus-naive and YF-immune monkeys. Three groups of 4 monkeys each were used. None of the monkeys showed any titer of neutralizing antibodies to YF or D2 at day 0 (Table 4). Groups 1 and 2 received one

Table 3. Immunogenicity of 17D-D2 virus in naive and YF-immune rhesus monkeys (Experiment 1).

Group	Monkey	$PRNT_{50}$			Challenge	
		0[a]	YF[b]	D2[c]	Viremia[d]	$PRNT_{50}$ to D2 (day 30)
1	L18	1:6	<1:5	1:731	0/0	1:4677
	M22	1:6	<1:5	1:1310	0/0	1:2005
	151	<1:5	<1:5	1:63	2.93/4	1:3236
2	R33	1:6	1: 25	1:105	3.78/4	1:3099
	R41	1:5	1:158	1:81	3.97/5	1:5662
	151^A	1:5	1:126	1:407	0/0	1:620
3	S51	1:7	1:316	<1:5	3.09/6	1:1422
	R59	1:10	1:501	<1:5	3.02/5	1:904
	165	1:5	1:630	<1:5	3.74/5	1:747
4	S19	1:5	<1:5	<1:5	3.28/4	1:1135
	R55	<1:5	<1:5	<1:5	2.2/4	1:1010
	185	<1:6	<1:5	<1:5	2.31/4	1:686

[a]PRNT for dengue 2 (D2) and yellow fever (YF) prior to any inoculation; [b]PRNT for YF at day 30 after YF vaccination; [c]PRNT for D2 virus at day 30 after inoculation of 17D-D2 virus; [d]peak viremia titer (log_{10} PFU/mL)/total number of days of viremia. PFU = plaque-forming units; PRNT = plaque reduction neutralization titer; $PRNT_{50}$ = neutralizing antibody titer or 50% PRNT.

Table 4. Immunogenicity of 17D-D2 virus in naive and YF-immune rhesus monkeys (Experiment 2).

Group	Monkey	$PRNT_{50}$			Challenge	
		Pre[a]	YF	D2[b]	Viremia[e]	$PRNT_{50}$ to D2 (day 30)
1	U11	<1/5	>1/630[c]	188	2.54/4	25988
	U45	<1/5	1/501	126	3.91/5	29854
	U59	<1/5	1/257	2131	1.40/3	45748
	U67	1/5	1/135	51	3.20/5	28454
2	U23	1/5	1/240[d]	71	4.54/5	14894
	U63	<1/5	1/389	2641	<0.7/0	4769
	U69	<1/5	>1/630	96	4.22/6	24618
	U75	<1/5	1/288	65	3.35/5	51531
3	U47	<1/5	<1/5	288	<0.7/0	57361
	U55	<1/5	<1/5	8925	<0.7/0	3099
	U57	<1/5	<1/5	4624	<0.7/0	4266
	U65	<1/5	<1/5	6090	<0.7/0	3393

[a]$PRNT_{50}$ for dengue 2 (D2) and yellow fever (YF) prior to any inoculation; [b]PRNT for D2 virus 60 days after inoculation with 17D-D2 virus, prior to challenge; [c]PRNT values at day 30 after YF vaccination, prior to 17D-D2 inoculation; [d]PRNT values at day 120 after YF vaccination, prior to 17D-D2 inoculation; [e]Peak viremia titer/total number of days of viremia. For abbreviations, see legend to Table 3.

human dose of YF 17DD virus *sc* and all developed antibodies to YF at 30 days *pi*. Group 1 received the 17D-D2 virus 30 days after immunization with YF 17DD virus and was challenged with D2 44-2 virus 60 days after receiving the 17D-D2 dose. None of the animals were protected, all of them showing high-post-challenge viremia titers. Monkey U59 showed a very high antibody titer to D2 (1:2131) but was not protected. However, peak viremia caused by the challenge virus was lower compared to the other monkeys in this group (Table 4). Group 2 was given the 17D-D2 virus 120 days after YF immunization and was challenged 60 days later. Only one animal (U63), which showed the highest titer of neutralizing antibodies to D2 in the group, was protected from viremia after challenge. In group 3, the YF-non-immune animals had measurable antibody titers to D2 60 days after immunization with the 17D-D2 chimera (Table 4). All animals were protected from challenge with D2 44-2. Animal U47 showed a lower neutralizing antibody titer (1:286) compared to the other animals but was also protected (Table 4).

Attenuation of 17D-D2 virus

The magnitude of post-inoculation viremia caused by the YF 17DD vaccine strain and the 17D-D2 chimeric virus after *sc* inoculation is a sensitive indicator of attenuation reflecting viral viscerotropism. In experiment 1, 4 of 6 animals inoculated with YF 17DD virus developed measurable viremia with titers ranging from 1.2 to 2.43 $\log_{10}$ PFU/mL on 1-6 days *pi*. Viremia was not detectable after the 6th day *pi*. Of the 6 monkeys that received the 17D-D2 virus, 2 showed minimal detectable titers of viremia (1.2 $\log_{10}$ PFU/mL) for 1 day only in the 10-day interval when blood samples were obtained (Table 5). One of these animals was YF-immune, and the other, YF-non-immune.

In experiment 2, of 8 animals inoculated with the YF 17DD vaccine, four animals had detectable viremia on the 4th day with a peak titer of 1.9 $\log_{10}$ PFU/mL and one animal (U63), on the 6th day (2.33 $\log_{10}$ PFU/mL). Only 2 of 12 animals that received the 17D-D2 virus developed detectable viremia (1.2 $\log_{10}$ PFU/mL). One of these was YF-immune (monkey U75 viremic on two consecutive days) and the other, non-immune (Table 5).

In both experiments it was evident that upon *sc* inoculation of similar doses, the 17D-D2 virus caused significantly lower levels of viremia compared to the YF 17DD vaccine virus. There was no apparent effect of prior YF-immunity on 17D-D2 viremia.

Table 5. Viremia of 17DD and 17D-D2 viruses after subcutaneous inoculation of rhesus monkeys.

	Viremia (17 DD)		Viremia (17D-D2)	
	Range ($\log_{10}$ PFU/mL)	Total number of viremic days	Range ($\log_{10}$ PFU/mL)	Total number of viremic days
Experiment 1				
Group 1	-	-	1.2	1
Group 2	1.2-2.43	8	1.2	1
Group 3	1.2	3	-	-
Group 4	-	-	-	-
Experiment 2				
Group 1	0.6	3	<0.7	-
Group 2	1.6-2.33	2	1.2	2
Group 3	-	-	1.2	1

PFU = plaque-forming units.

Low neurovirulence of the 17D-D2 chimera

Further evidence for the attenuation of the 17D-D2 virus was obtained by neurovirulence testing in monkeys using the YF 17DD vaccine as control. A total of 10 monkeys for each virus were inoculated intracerebrally (*ic*) and the animals were observed for 30 days for clinical signs, after which they were sacrificed and their central nervous system (CNS) and several other organs were removed for histological analyses. Individual scores for each of the 20 monkeys used in the test are shown in Table

6. Only 2 of the 20 monkeys displayed signs of encephalitis (V9 and V75), both inoculated with the YF 17DD virus. For the 17D-D2 virus, only 2 monkeys (V08 and V51) displayed minor clinical signs (anorexia and a rough coat) resulting in an overall clinical score of 0.03 for the virus. The YF 17DD virus induced somewhat more intense clinical signs in 3 monkeys (V9, V11 and V75) with a fourth monkey showing minor clinical signs (V79). The clinical score for the chimeric virus was about one third that for the reference vaccine virus but the difference was not statistically significant (P = 0.65, Kruskal-Wallis test).

None of the 20 animals displayed any anatomical or histological abnormality in any of the extra-neural organs analyzed which included tongue, salivary mandibular gland, heart, lung, liver, kidney, urinary bladder, mesenteric lymph node, axillary lymph node, spleen, stomach, duodenum, and colon. Despite intracerebral inoculation with the 17D-D2 virus, the monkeys did not show any evidence of clinical dengue infection nor were any specific lesions produced either at the site of injection or in the spinal cord, brain stem, thalamus, and frontal, parietal, temporal and occipital cortex. The appearance of specific microscopic lesions after inoculation of the YF 17DD virus indicated involvement of the CNS.

None of the 10 animals inoculated with the 17D-D2 virus showed lesions of grade 2 or higher in any of the 7 CNS areas studied and only 3 had grade 1 lesions in the substantia nigra. Another animal had grade 1 in the putamen. For the YF 17DD virus, grade 3 lesions were observed in two animals (V75 and V79) in the nucleus caudatus. Grade 2 lesions were noted in the substantia nigra of 7 animals and in one or more of the 7 discriminatory areas of 8 of 10 monkeys.

Only 3 monkeys inoculated with the 17D-D2 virus (V39, V55 and V89) displayed minor neuronal involvement in the target area (substantia nigra), yielding a mean score of 0.15 for this area. In contrast, all monkeys which received the YF 17DD vaccine did have lesions in the target area yielding a mean score of 1.5, as expected for this virus (21). The discriminatory areas of the CNS of monkeys inoculated with the 17D-D2 virus were minimally affected in only two monkeys (V08 and V61), yielding a mean score of 0.011, whereas all 10 monkeys that received the YF 17DD vaccine virus displayed some degree of neuronal involvement (mean score of 0.67). The combined histological score for the monkeys inoculated with the 17D-D2 virus was 0.08, whereas it was 1.07 for YF 17DD, a characteristic value for this virus (21). The statistical significance of the difference between means was evaluated by the nonparametric Kruskal-Wallis test since variance was too high (P = 0.0003, variance ratio test). The difference in the combined histological scores for both viruses was found

Table 6. Clinical and histological scores for the neurovirulence test in rhesus monkeys.

Virus	Monkey	Clinical score	Discriminatory areas	Target area	Combined histological score
17D-D2	V08	0.1	0.04	0	0.02
	V33	0	0	0	0
	V39	0	0	0.5	0.25
	V47	0	0	0	0
	V51	0.2	0	0	0
	V55	0	0	0.5	0.25
	V61	0	0.07	0	0.04
	V65	0	0	0	0
	V77	0	0	0	0
	V89	0	0	0.5	0.25
	Mean ± SD	0.03 ± 0.06[a]	0.011 ± 0.02	0.15 ± 0.02	0.081 ± 0.12[b]
17DD	V09	0	0.54	2	1.27
	V11	0.3	0.5	1.5	1
	V43	0	0.07	0.5	0.16
	V45	0	0.43	1	0.71
	V67	0	1.12	2	1.56
	V75	0.5	1.05	2	1.52
	V79	0.07	1.36	1.5	1.43
	V81	0	0.2	1	0.6
	V85	0	0.5	1.5	1
	V91	0	0.92	2	1.46
	Mean ± SD	0.087 ± 0.17[a]	0.67 ± 0.42	1.5 ± 0.52	1.07 ± 0.46[b]

[a]P = 0.65 for 17D-D2 *vs* 17DD (Kruskal-Wallis test); [b]P = 0.0004 for 17D-D2 *vs* 17DD (Kruskal-Wallis test).

to be highly significant (P = 0.0004), further confirming the attenuated nature of our 17D-D2 chimeric virus (Table 6).

Blood samples were also collected from these animals on days 2 through 6 *pi* for analysis of viremia. As described above for viremia after *sc* inoculation, the viremia levels of the 17D-D2 virus were clearly lower (peak titer of 0.96 log_{10} PFU/mL and mean duration of 0.6 days) compared to YF 17DD (mean peak titer of 1.69 log_{10} PFU/mL and mean duration of 3.1 days) after *ic* inoculation (Table 7). Taken together, the viremia data after both *sc* and *ic* inoculation of monkeys demonstrated limited viral replication, particularly for the chimera.

Table 7. Viremia in monkeys inoculated intracerebrally with 17DD and 17D-D2 viruses.

Virus	Monkey	Viremia (day)					Mean peak viremia	Total viremia days
		2nd	3rd	4th	5th	6th		
17DD	V09	<0.4	<0.4	0.9	<0.4	<0.4	1.69 ± 0.74	31
	V11	<0.4	1.24	0.88	<0.4	<0.4		
	V43	0.4	0.9	0.4	0.7	0.4		
	V45	1.0	2.24	1.78	1.54	<0.4		
	V67	<0.4	<0.4	1.18	1.18	<0.4		
	V75	1.48	2.83	2.59	1.2	<0.4		
	V79	1.24	2.43	2.07	1.35	<0.4		
	V81	0.4	2.06	2.01	0.4	<0.4		
	V85	<0.4	<0.4	<0.4	<0.4	<0.4		
	V91	0.4	0.4	0.88	0.4	0.4		
17D-D2	V08	0.4	<0.4	<0.4	<0.4	<0.4	0.96 ± 6	6
	V33	<0.4	<0.4	<0.4	<0.4	<0.4		
	V39	<0.4	<0.4	<0.4	<0.4	<0.4		
	V47	<0.4	0.9	<0.4	<0.4	<0.4		
	V51	<0.4	<0.4	<0.4	<0.4	<0.4		
	V55	0.4	<0.4	<0.4	0.4	<0.4		
	V61	<0.4	<0.4	<0.4	<0.4	<0.4		
	V65	<0.4	1.97	<0.4	<0.4	<0.4		
	V77	1.68	<0.4	<0.4	<0.4	<0.4		
	V89	<0.4	<0.4	<0.4	<0.4	<0.4		

Table 8. Differences in amino acid sequence between ChimeriVax-D2 and 17D-D2 viruses.

Gene (amino acid)	Virus	
	17D-D2	ChimeriVax-D2[a]
prM/M 125[b]	Threonine	Isoleucine
E 141	Isoleucine	Valine
E 164	Isoleucine	Valine
E 308	Isoleucine	Valine
NS5 391[c]	Serine	Asparagine
NS5 657	Asparagine	Aspartic acid

[a]The ChimeriVax-D2 sequence was derived by splicing together the prM/E genes from dengue-2 (D2) type virus PUO 218 strain (GenBank accession number D00345) and the yellow fever backbone based on the YF 17D sequence of Rice et al. (9) (GenBank accession number X03700) and introducing the nucleotide sequence differences reported by Guirakhoo et al. (16). [b]Numbering based on D2 virus NGC strain (GenBank accession number M29095). [c]Numbering based on YF 17D sequence (GenBank accession number X03700).

Nucleotide sequence analysis of the 17D-D2 virus

The 17D-D2 virus at passage 2 had its genome entirely sequenced. The extreme 5' and 3' ends were sequenced on one strand only. The genome consists of 10,874 nucleotides. We detected 3 nucleotide changes at passage 2 virus genome as compared to the cDNA sequence in the full-length infectious clone at positions 1765 (G→A), 2170 (C→T) and 10,467 (G→A), all of which were silent.

Given that our 17D-D2 virus and the ChimeriVax-D2 virus (16) have their prM/E genes derived from different D2 strains, it was relevant to compare their respective amino acid sequences (Table 8). The D2 moiety in the FIOCRUZ 17D-D2 viral genome consists of D2 New Guinea C (NGC) sequences from nucleotide 432 (numbering according to the D2 NGC sequence, GenBank accession number M29095) to nucleotide 1715 where an *Nsi*I site was created for fragment exchange. From this position to nucleotide 2414 (the signalase cleavage site between E and NS1), the cDNA used for the construction came from a Brazilian isolate of D2 virus (13). Throughout the prM/E region, the 17D-D2 virus differs from ChimeriVax™-D2 at 89 nucleotide positions, leading to 4 amino acid substitutions (Table 8).

In the YF moiety, the 17D-D2 virus differs at 4 nucleotide positions in relation to

ChimeriVax-D2: 6898 (NS4B, silent), 8656 (NS5, silent), 8808 (NS5 391/S→N), and 9605 (NS5 657/N→D; Table 8). No heterogeneous positions were noted in the YF moiety of the chimeric virus.

Discussion

In a previous report we described the construction of a chimeric 17D-D2 virus through the exchange of the prM/E genes. This virus was characterized in terms of *in vitro* growth, immunogenicity and attenuation for mice (13). Here, we have further extended these studies to non-human primates.

We examined the ability of the 17D-D2 virus to induce protective immune responses in flavivirus-naive rhesus monkeys. Altogether, 6 of 7 naive monkeys immunized with 17D-D2 (in both experiments 1 and 2) developed neutralizing antibodies to D2 and were protected from challenge. The neutralizing titers to D2 significantly increased after challenge, suggesting an anamnestic response. In contrast, animals that received the YF 17DD vaccine or medium only showed consistent viremia after wild-type D2 virus challenge and seroconverted to D2 thereafter.

The question of pre-immunity to YF is of importance in validating the use of YF-based recombinant vaccines in areas with intense vaccination for YF, as is the case for many South American countries. In experiment 1, 3 animals received a human dose of YF 17DD vaccine by the *sc* route followed by a single dose of 17D-D2 virus 30 days later. All 3 animals seroconverted to YF. Only animal 151^{A} had a significant neutralizing antibody titer to D2 and was protected. The other two animals (R33 and R41) had lower anti-D2 antibody levels prior to challenge and were not protected suggesting that pre-immunity to YF interfered with immunization by the 17D-D2 virus. Guirakhoo et al. (16) reported that anti-YF 17D immunity did not preclude induction of immunity and protection by the ChimeriVax-D2 virus. These investigators used an interval of 120 days between YF and chimeric virus vaccinations. It is possible that the interference in experiment 1 was due to a shorter interval (30 days) between the YF and 17D-D2 immunizations. This possibility was addressed in experiment 2. Two groups of 4 monkeys received the YF 17DD vaccine 30 or 120 days prior to inoculation with the 17D-D2 virus. A third group received the chimera only. Neutralizing antibody titers to D2 were generally lower in the YF pre-immune groups as compared to the YF-naive group, with the exception of monkeys U59 and U63. The 6 animals that did not have high anti-D2 neutralizing antibody titers were not protected from challenge, while monkeys U59 and U63 were partially or completely protected (Table 4). All 4 naive monkeys given the chimera only were fully protected. No difference was observed for the time intervals of 30 and 120 days between YF 17DD and 17D-D2 virus vaccination. The YF-Vax and FIOCRUZ YF vaccines used for pre-immunization of monkeys in the two studies (16) and in this study are based on different substrains of the 17D virus, 17D-204 and YF 17DD, respectively. These substrains have been shown to differ in monkey neurovirulence (21), but whether they elicit somewhat different anti-YF immune responses in monkeys that would explain the differences in results from the two groups is unclear.

The ChimeriVax-D2 virus replicates efficiently in naive monkeys as judged by detectable viremia in most animals, while the levels of viremia are reduced in YF-immune animals (16). In our experiments, the viremia levels of the 17D-D2 virus were below the level of detection in most YF-immune and -non-immune monkeys (Table 5). The neutralizing antibody titers to D2 in monkeys immunized with the ChimeriVax-D2 virus tended to be higher in naive animals than in monkeys previously immu-

nized with the YF 17D virus. The neutralizing antibody titers elicited by the 17D-D2 virus in the YF-non-immune monkeys (geometric mean titer 1234) were significantly higher than in YF-immune animals (geometric mean titer 188; P = 0.05, Kruskal-Wallis test). The apparent limited replicative capability of our 17D-D2 virus could provide an explanation for its inability to solidly immunize monkeys against D2 in the presence of immunity to YF.

Most flavivirus proteins elicit some type of immune responses, either humoral or cellular (22). There is evidence in the literature that immunity to YF NS1 protein protects mice (23,24) and monkeys (25) from an otherwise fatal challenge. Studies on human cytotoxic T lymphocyte responses to live attenuated YF 17D vaccine led to the identification of HLA-B35-restricted cytotoxic T lymphocyte epitopes on nonstructural proteins NS1, NS2B and NS3 (26). van der Most et al. (27) have shown that the YF 17D virus envelope and NS3 proteins are major targets of the antiviral T cell response in mice. Since the chimeric virus contains eight of the YF virus proteins it is conceivable that immunity to any of those may compromise chimeric virus replication in the YF immune host, thereby reducing the response to the dengue proteins and resulting in the lower level of protection observed in experiments 1 and 2.

One of the hallmarks of the YF 17D vaccine is its extremely low incidence of adverse events. A total of 21 cases of neurologic disease have been reported (2) among millions of vaccinees and encephalitic reactions were also observed in the early development of the YF 17D virus (28). Since neurotropism is a concern with YF 17D vaccines, a standardized neurovirulence test in monkeys was established to ensure the attenuation of any YF 17D virus (29,30). Although dengue in general is known to be non-neurovirulent (31) recent reports have indicated cases of encephalitis after natural dengue infection (32-34). Because YF 17D-dengue chimeric viruses contain the replicative machinery of YF 17D virus and the envelope genes of dengue viruses, their safety should be assessed by the formal neurovirulence test. Other attenuated dengue candidate vaccine viruses have also been tested in the same manner (35-39) before entering clinical trials. Here we demonstrated that the 17D-D2 virus caused lower viremia of shorter duration after *ic* inoculation as compared to the YF 17DD vaccine control. The viremias of both viruses were within the WHO limit of less than 100 LD_{50}/0.03 mL of serum. The peak viremia of our 17D-D2 chimera was lower than that observed for ChimeriVax-D2 virus inoculated into the CNS of rhesus monkeys in a similar test but using a mixture of all 4 17D-dengue chimeric viruses (ChimeriVax-dengue types 1 through 4 (15).

Vaccine-related clinical signs were observed in 4 of 10 monkeys that received YF 17DD virus beginning at day 8 *pi* and included encephalitis (monkeys V9 and V75). Clinical signs were observed in only 1 monkey of 10 that received the 17D-D2 virus and were minor. Accordingly, the clinical score for the 17D-D2 group was lower than that for the YF 17DD group. Likewise, the histological lesions in both target and discriminatory areas of monkeys inoculated with the 17D-D2 virus were less frequent, with fewer neuronal changes and yielding a combined histological score significantly lower than that observed for the control YF 17DD vaccine virus ($P < 0.0004$). These data, together with the observed limited viremia, demonstrate the high attenuation of our 17D-D2 virus, similar to that of the recently described tetravalent ChimeriVax-dengue vaccine (15).

A total of 93 nucleotide sequence differences and 6 amino acid substitutions have been detected between our 17D-D2 and ChimeriVax-D2 viruses. Although they seem to replicate with the same efficiency in Vero cells, reaching 7.0 log_{10} PFU/mL, the observed nucleotide sequence differences may

be a burden for the former virus to replicate in rhesus monkeys.

It is generally accepted that a dengue vaccine should be tetravalent. The attenuation profile for monkeys as well as the immunogenicity and protective efficacy of the 17D-D2 virus lend further support to the development of a tetravalent dengue vaccine using chimeric 17D-dengue viruses. However, the data shown here suggest that the prM/E sequences used for the construction of chimeras may influence vaccine performance. In this regard a more immunogenic 17D-D2 variant may need to be produced, which replicates better in the primate model, and then tested in a tetravalent vaccine formulation.

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